

Editor's Summary

Potentiating Trouble

Cystic fibrosis (CF) is a genetic disease caused by mutations of the CF transmembrane conductance regulator (CFTR) ion channel, resulting in pulmonary and other complications. Ivacaftor is the only targeted drug approved for CF, but it is not effective enough to treat the severest and most common form of this disease. Ivacaftor is a "potentiator," which means that it improves the activity of mutant CFTR but cannot work if there is no CFTR on the cell surface. Other drugs, called "correctors," help bring mutant CFTR to the cell surface, but two manuscripts by Cholon and Veit and coauthors now show that combining the two types of drugs does not work effectively, because potentiators make CFTR less stable, accelerating the removal of this channel from the cell membrane.

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CYSTIC FIBROSIS

Potentiator ivacaftor abrogates pharmacological correction of $\Delta F508$ CFTR in cystic fibrosis

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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, $\Delta F508$. Thus, patients with $\Delta F508$ 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 $\Delta F508$ homozygous cultures. This result reflected the destabilization of corrected $\Delta F508$ CFTR by VX-770, markedly 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 these drugs to maximize rescue of $\Delta F508$ 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, $\Delta F508$ 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 two groups: (i) “corrector” compounds that promote maturation and delivery of CFTR proteins to the apical surface and (ii) “potentiator” compounds that activate apical CFTR by increasing the open time of the channel.

The U.S. Food and Drug Administration 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 adenosine triphosphate (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).

Approximately 90% of CF patients carry the $\Delta F508$ 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 $\Delta F508$ mutation (23), likely because this compound only acts on protein that has trafficked to the plasma membrane. On the basis of these findings, an attractive therapeutic strategy for the $\Delta F508$ CF patient population is to promote transfer of the endoplasmic reticulum (ER)-retained $\Delta F508$ CFTR protein to the plasma membrane using small-molecule corrector compounds (24–26). Studies have estimated that the extent of correction in $\Delta F508$ airway epithelial cells must approximate 10 to 25% of wild-type CFTR function to provide therapeutic benefit (27, 28). In vitro treatment of CF airway epithelial cultures homozygous for the $\Delta F508$ 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 $\Delta F508$ CF patients in recent clinical trials, most likely because $\Delta F508$ CFTR correction in vivo was less than 10% of wild-type levels, the lower limit of detection, and thus, no mature $\Delta F508$ CFTR protein was observed (29). Therefore, a logical next step was to combine corrector

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and potentiator therapies to rescue $\Delta F508$ and increase protein function (24, 30, 31). One of the most promising current clinical trials designed to optimize $\Delta F508$ CFTR function involved the administration of the corrector VX-809 with the potentiator VX-770. Increases in VX-809-rescued $\Delta F508$ 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 ($\Delta F508/\Delta F508$) intestinal tissue (32). Surprisingly, chronic coadministration 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 $\Delta F508$ CFTR mutation (31, 33).

The aim of this study was to elucidate the molecular mechanism(s) underlying the limited improvement in $\Delta F508$ CFTR function when a corrector, VX-809, and a potentiator, VX-770, were coadministered 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 was used to investigate this interaction. Human bronchial epithelial (HBE) cells were used for these studies and exposed for 48 hours 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 used to explore the effects of VX-770 on wild-type 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 HBE cells derived from CF patients with the *G551D* mutation on one allele and the $\Delta F508$ 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/\Delta F508*) as a model. Cultures were treated chronically for 48 hours 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 to forskolin to stimulate Cl^- secretion by CFTR. As previously reported, acute administration of VX-770 (aVX-770) raised Cl^- secretion after forskolin administration (Fig. 1A). Chronic VX-770 (cVX-770) administration raised forskolin responsiveness but eliminated subsequent responses to acute VX-770 administration (Fig. 1, A to C, and 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/\Delta F508* HBE cultures also exhibited a decrease in amiloride-sensitive currents (fig. S1A and 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

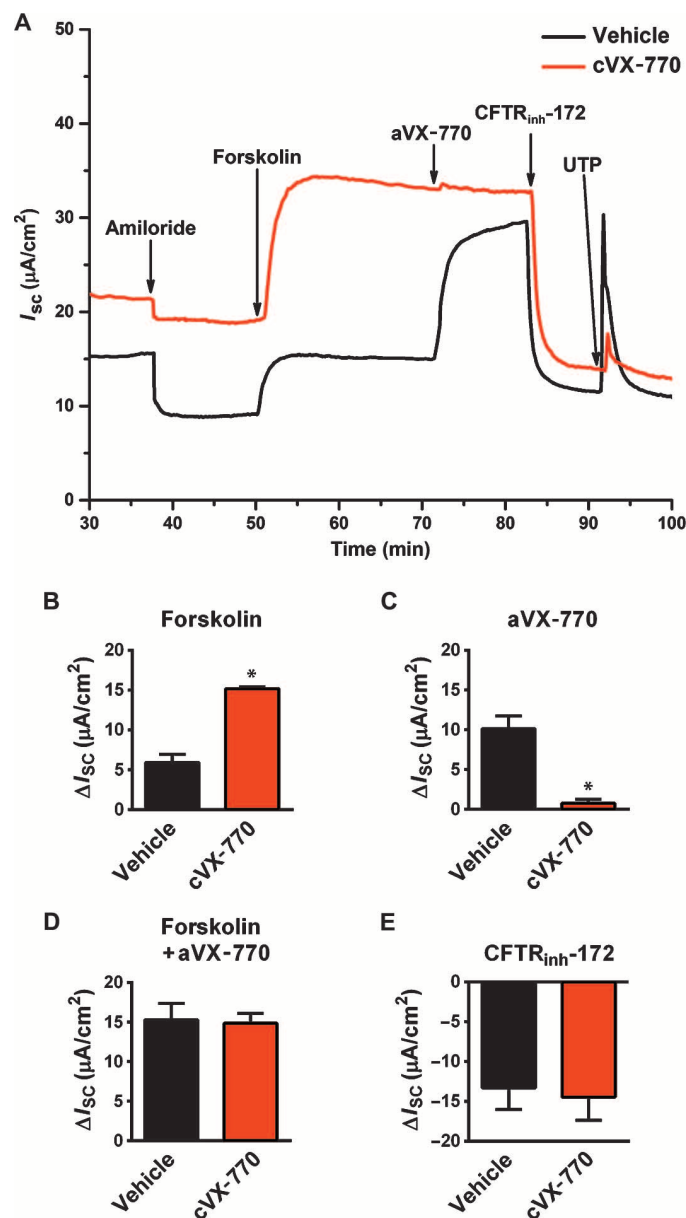


Fig. 1. VX-770 treatment restores *G551D* function. Electrophysiological properties of *G551D/\Delta F508* cultures analyzed in Ussing chambers treated chronically with VX-770 (cVX-770; 5 μM for 48 hours) or with vehicle [0.1% dimethyl sulfoxide (DMSO)]. (A) Representative recording of I_{sc} measured in Ussing chambers. (B to E) Quantification of response to treatment with forskolin (significant difference between vehicle and cVX-770, * $P = 0.0009$) (B), acute VX-770 (aVX-770) (significant difference between vehicle and cVX-770, * $P = 0.0054$) (C), forskolin + aVX-770 (D), and CFTR_{inh}-172 (E). Primary CF HBE cultures (*G551D/\Delta F508*) were derived from three different patients, and three to four replicates were performed per patient for a total of 10 measurements per treatment.

cultures (fig. S1B). The average uridine triphosphate (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 and 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 $\Delta F508$ CFTR

CF patients harboring the $\Delta F508$ 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 $\Delta F508$ CFTR. To mimic clinical administration of VX-809 and VX-770 as a combination therapy for the $\Delta F508$ CF patients, primary CF HBE cultures ($\Delta F508/\Delta F508$) were treated with both pharmacological agents for 48 hours, and then Ussing chamber experiments were performed to measure $\Delta F508$ CFTR function (Fig. 2 and table S3).

Anion efflux across the apical epithelial membrane of airway epithelia in response to cyclic adenosine monophosphate (cAMP) is mediated by CFTR and was not substantial in vehicle-treated $\Delta F508/\Delta F508$ CF HBE cultures (Fig. 2, A and B; Vehicle). However, VX-809 administration produced $\Delta F508$ CFTR correction as evidenced by stimulation of Cl^- secretion (Fig. 2). Specifically, correction by VX-809 produced responses to forskolin (Fig. 2, A and B; VX-809) that were enhanced by acute administration of VX-770 (Fig. 2, A and C). These data indicate that acutely applied VX-770 further activated (“potentiated”) VX-809–rescued $\Delta F508$ CFTR. However, the CFTR-mediated I_{SC} increase after addition of VX-770 to corrector-rescued $\Delta F508$ 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 $\Delta F508$ CFTR function was markedly decreased in cultures that had been chronically treated with VX-809 and VX-770 compared to VX-809 alone (Fig. 2A, VX-809 versus VX-809 + cVX-770). 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 (Fig. 2A and fig. S1D).

We also tested the impact of chronic VX-770 treatment on $\Delta F508$ correction in CF HBE cultures ($\Delta F508/\Delta F508$) by the 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 and table S4).

Chronic VX-770 administration hinders correction by decreasing the stability of corrected $\Delta F508$ CFTR

To explore the mechanism(s) mediating the VX-770–induced reduction of VX-809–corrected $\Delta F508$ 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 $\Delta F508/\Delta F508$ CF HBE cells (Fig. 3A, CF). As previously reported, treatment with VX-809 alone resulted in the 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 $\Delta F508$ CFTR was diminished, and instead, the

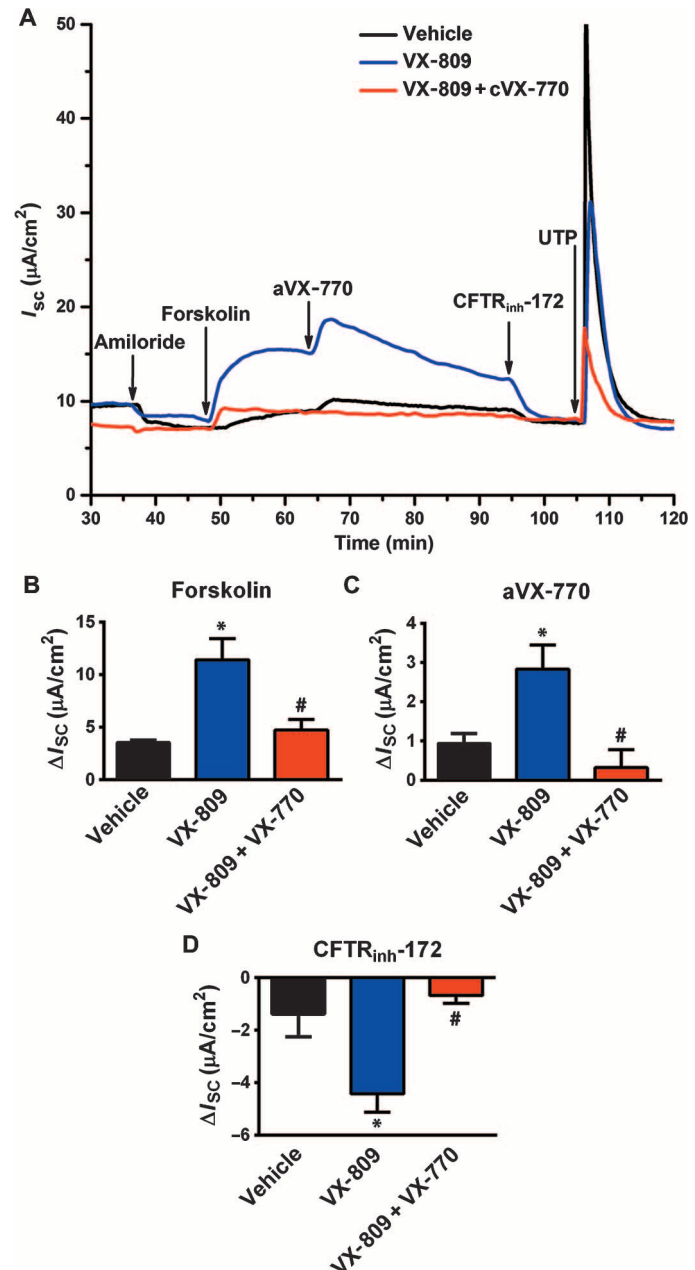


Fig. 2. Chronic VX-770 treatment inhibits functional rescue of $\Delta F508$.

(A) Representative I_{SC} traces of CF HBE cells recorded in Ussing chambers. Primary CF HBE cells ($\Delta F508/\Delta F508$) were treated with vehicle (DMSO) or VX-809 \pm VX-770 for 48 hours at 5 μM each. (B) ΔI_{SC} response to forskolin observed in VX-809–treated CF HBE cells (* $P = 0.0033$, VX-809 versus vehicle) was prevented by chronic VX-770 treatment and significantly different from VX-809–treated cells (# $P = 0.0147$, VX-809 versus VX-809 + VX-770). (C) CF HBE cells treated with VX-809 responded to acute VX-770 exposure (* $P = 0.0177$, VX-809 versus vehicle). This response was significantly abrogated in VX-809 + VX-770–treated cells (# $P = 0.0031$, VX-809 versus VX-809 + VX-770). (D) The response to CFTR_{inh}-172 observed in VX-809–treated cells (* $P = 0.0209$, VX-809 versus vehicle) was significantly decreased in VX-809 + VX-770–treated cells (# $P = 0.0006$, VX-809 versus VX-809 + VX-770). Primary HBE cultures ($\Delta F508/\Delta F508$) were derived from six different patients, and two to four replicates were performed per patient for a total of 15 measurements per condition.

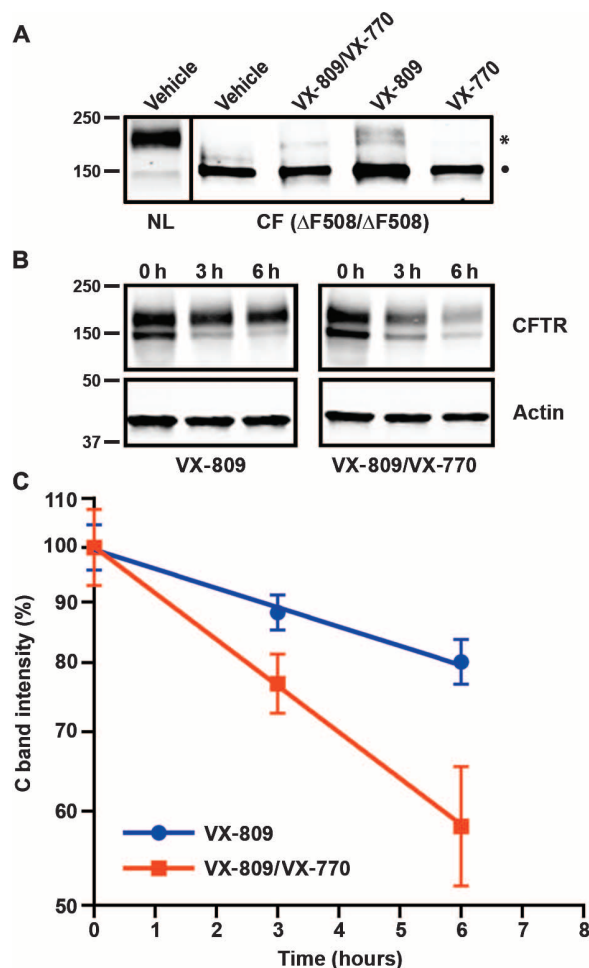


Fig. 3. VX-770 diminishes biochemical correction by increasing turnover of corrected $\Delta F508$ CFTR. (A) CFTR Western blot of normal (NL) and CF HBE cultures treated with VX-809 (5 μM) \pm VX-770 (5 μM) for 48 hours. “*” indicates the mature, complex glycosylated form of CFTR, band C; “•” indicates the immature band B. (B) Turnover of rescued $\Delta F508$ in BHK-21 cells. $\Delta F508$ was rescued at 27°C in the presence of VX-809 \pm VX-770 for 24 hours. After adding cycloheximide (200 $\mu g/ml$, 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 ($n = 3$).

$\Delta F508$ CFTR protein appeared almost exclusively as immature band B. These data suggest that chronic VX-770 treatment impeded correction of $\Delta F508$ CFTR by VX-809. We investigated the impact of chronic VX-770 treatment on protein stability by measuring the turnover rate of corrected $\Delta F508$ CFTR. $\Delta F508$ 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 hours because VX-770 prevented VX-809-mediated correction of $\Delta F508$ 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 $\Delta F508$ CFTR was then measured after 3 and 6 hours. The turnover rate of rescued $\Delta F508$ CFTR band C increased, and the half-life accordingly decreased by ~ 2.5 -fold in the presence of VX-770 (Fig. 3, B and C, and table S5), whereas the decrease

in band B levels was not affected by the presence of VX-770 (Fig. 3B). These data show that VX-770 decreased the stability, and thus increased the turnover rate, of VX-809-rescued $\Delta F508$ CFTR.

VX-770 affects correction of $\Delta F508$ CFTR in a dose-dependent relationship

Lower concentrations of VX-770 were chronically administered to $\Delta F508/\Delta F508$ CF HBE cells to study the dose effect on VX-809-rescued $\Delta F508$ 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. 4, A and B, and 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 and table S7). Chronic treatment with either 50 nM or 1 μM VX-770 eliminated responses to acute VX-770 (Fig. 4, A and 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 $\Delta F508$ CFTR decreased (Fig. 4, D and E, and table S8).

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

To investigate the effects of chronic VX-770 treatment on normal CFTR, we measured anion secretion of normal primary HBE cultures treated with VX-770 for 48 hours (Fig. 5). Strikingly, administration of 5 μM VX-770 reduced CFTR-mediated Cl^- secretion, as reflected by decreased forskolin responses (Fig. 5, A to C, and table S9) and decreased inhibition of CFTR-mediated current by CFTR_{inh}-172 (Fig. 5, A and 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 and table S2) and a substantial inhibition of UTP-sensitive current (fig. S3B and table S2) similar to that observed in CF HBE cells (fig. S1, A, C, and D, and 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 of primary HBE cultures was also not affected by chronic VX-770 exposure (Fig. 6B and 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 (Na^+ - and K^+ -dependent adenosine triphosphatase) 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

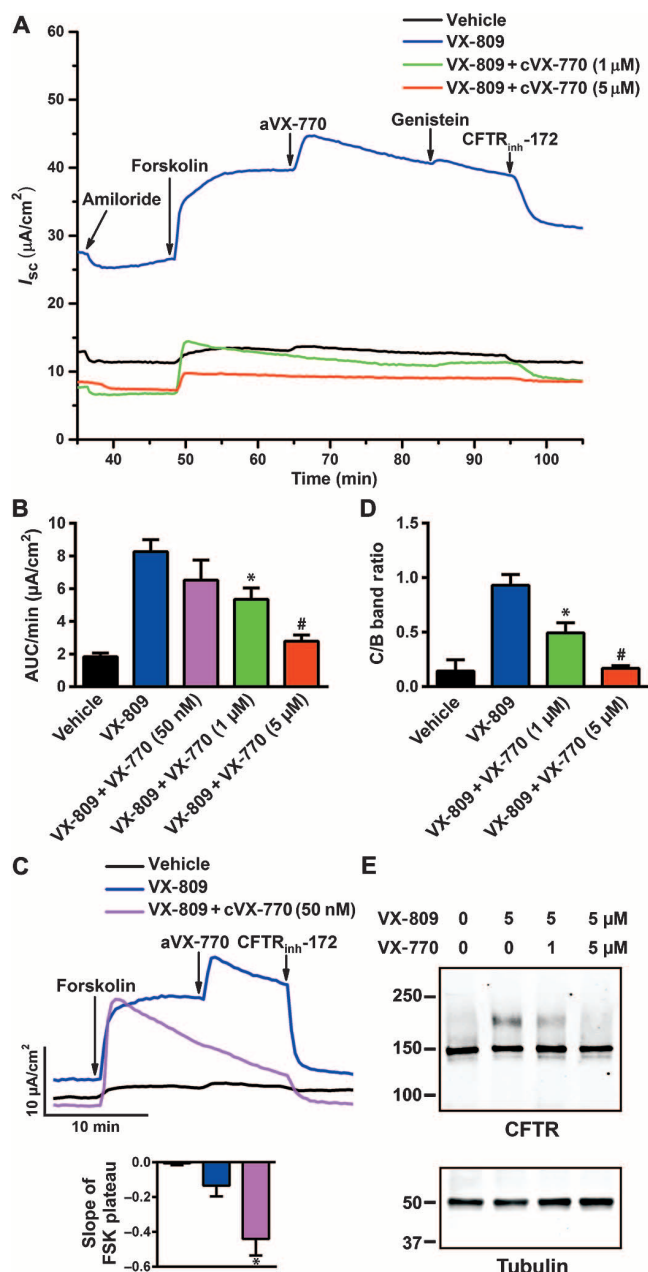


Fig. 4. VX-770-induced hindrance of $\Delta F508$ correction is dose-dependent. (A) I_{sc} traces of CF HBE cells ($\Delta F508/\Delta F508$) recorded in Ussing chambers. CF HBE cells were treated as indicated (VX-809, 5 μM ; VX-770, 1 or 5 μM) for 48 hours. (B) CFTR function in VX-809-treated cells decreased as chronic VX-770 concentrations increased. Significant reduction of the 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 hours 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 versus VX-809 + 5 μM VX-770). Primary CF HBE cultures were derived from at least four different CF patients, and two to five replicates were performed per patient for a total of at least 14 measurements per condition. (C) In VX-809-corrected CF HBE cultures ($\Delta F508/\Delta F508$), the presence of chronic VX-770 at 50 nM caused a significant decline of the slope after forskolin (FSK) treatment (* P = 0.0353, VX-809 versus VX-809 + 50 nM VX-770). Primary CF HBE cultures were derived from four different patients, and three to five 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 ($\Delta F508/\Delta F508$). 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 hours 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 to 5 μM (* P = 0.0151, VX-809 + 1 μM VX-770 versus VX-809 + 5 μM VX-770). Primary CF HBE cultures ($\Delta F508/\Delta F508$) from four different patients were analyzed. (E) Representative Western blot of CF HBE cells ($\Delta F508/\Delta F508$) showing decrease of VX-809-corrected $\Delta F508$ as chronic VX-770 concentrations were increased.

rescued $\Delta F508$ 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 normal cultures (Fig. 7B, NL, and table S12). In contrast, the levels of mature (band C) G551D CFTR detected in *G551D/\Delta F508* 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/\Delta F508*).

To explore the relationship between VX-770, VX-809, and CFTR protein stability, we performed calculations of thermodynamic stability of CFTR protein using 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 $\Delta F508$ CFTR, the deletion of amino acid F508 not only reduces the stability of the NBD1 domain but also, importantly, may destabilize multidomain assembly of CFTR (39–41). In contrast, in this structural model of CFTR, amino acid G551 is positioned between the two NBDs (Fig. 7C and fig. S5), and the *G551D* mutation is thought to contour 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 $\Delta\Delta G$ for *G551D* CFTR (43, 44). We found that *G551D* had a stabilizing effect on the CFTR protein ($\Delta\Delta G$ = -8.1 kcal/mol).

vehicle- or VX-770-treated HBE cultures (Fig. 6C and table S10), suggesting intact Na^+, K^+ -ATPase activity (37). Measurements of intracellular concentrations of VX-809 and VX-770 by mass spectrometry (MS) 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 and table S11).

Destabilization by VX-770 is beneficial for G551D, but not for wild-type, or $\Delta F508$ CFTR function

A high C/B band ratio indicates normal CFTR protein maturation. Biochemical analysis by Western blotting showed that chronic VX-770 treatment markedly reduced the amount of mature CFTR in both normal cultures expressing wild-type CFTR (Figs. 5E and 7A) and VX-809–

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*

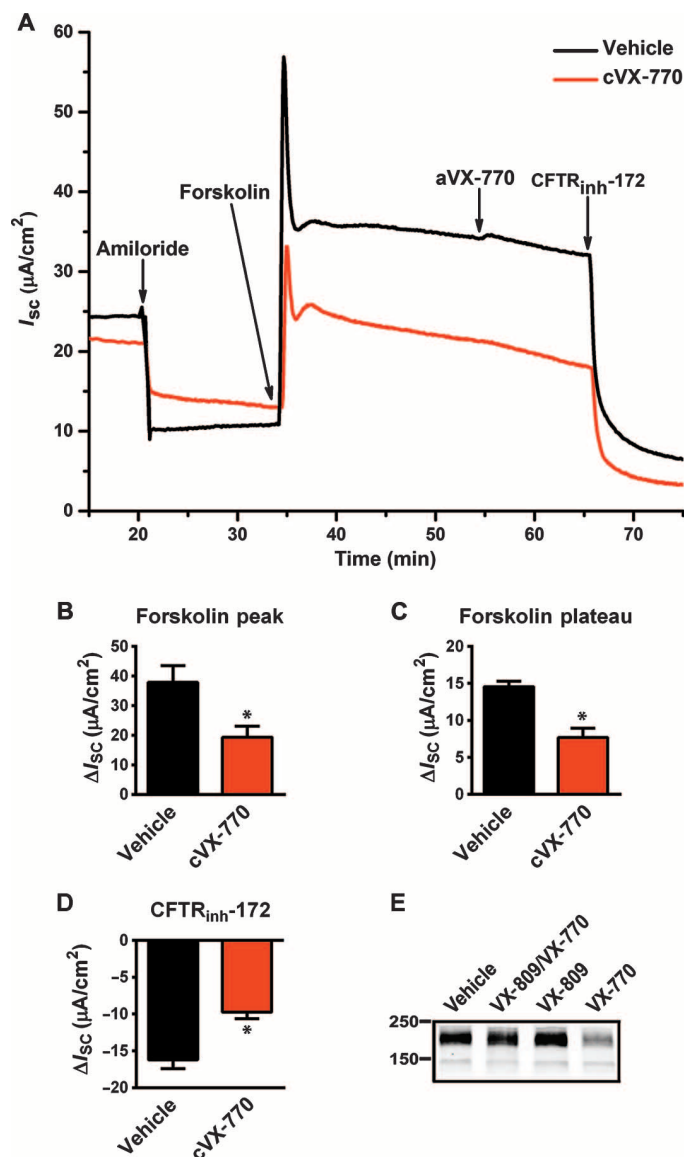


Fig. 5. Chronic VX-770 treatment decreases function of wild-type CFTR. (A) Representative I_{sc} traces of normal HBE cells recorded in Ussing chambers. Cultures were treated with vehicle (DMSO) or 5 μM VX-770 for 48 hours. (B to D) HBE cells that were chronically treated with VX-770 showed significantly reduced response to forskolin ($*P = 0.0198$ for forskolin peak and $*P = 0.0008$ for forskolin plateau) (B and C) and CFTR_{inh}-172 ($*P = 0.0014$) (D). Primary HBE cultures were derived from six different individuals, and two to four 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) \pm VX-770 (5 μM) for 48 hours. Mature CFTR was diminished in HBE cells that were chronically treated with VX-770.

mutation. However, the most common mutant protein in CF patients, $\Delta F508$ CFTR, is not found at the cell surface. $\Delta F508$ 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 $\Delta F508$ proteins to the cell surface. Corrector-rescued $\Delta F508$ CFTR is reported to have a shorter half-life at the cell surface (45–49) and ex-

hibits increased thermal inactivation as well as a gating defect when compared to wild-type 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*/ $\Delta F508$ 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*/ $\Delta F508$ 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 β_2 -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 about 15% of normal function in $\Delta F508$ / $\Delta F508$ CF HBE cells (8). However, 10 to 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 $\Delta F508$ function may be necessary. Although acute treatment with VX-770 has been reported to enhance VX-809-rescued $\Delta F508$ activity (8), our data revealed that chronic application of VX-770 in combination with VX-809 or VX-661 did not. Chronic coadministration of VX-770 with either corrector to $\Delta F508$ / $\Delta F508$ 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 $\Delta F508$ CFTR. The VX-770-induced reduction of $\Delta F508$ 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 $\Delta F508$ CF HBE cells do not predict the outcome for chronic VX-770/VX-809 administration.

$\Delta F508$ 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 $\Delta F508$ CFTR by enhancing interactions among NBD1, MSD1, and MSD2 (60, 61). Thus, VX-809 is predicted to enhance function of $\Delta F508$ by increasing its stability (Fig. 7D). Chronic exposure to VX-770 appeared to reverse the stabilization effect of VX-809 in $\Delta F508$ CFTR. Chronic VX-770 treatment resulted in a severe reduction in rescued $\Delta F508$ CFTR protein due to destabilization of rescued protein as reflected by a 2.5 \times 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 wild-type or $\Delta F508$ CFTR.

Our CFTR computational structural model (38) allows us to speculate how VX-770 may interact with wild-type 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 are finely tuned and precisely balanced, which is a requirement for its ability to function properly as a regulated ion channel. The inherent

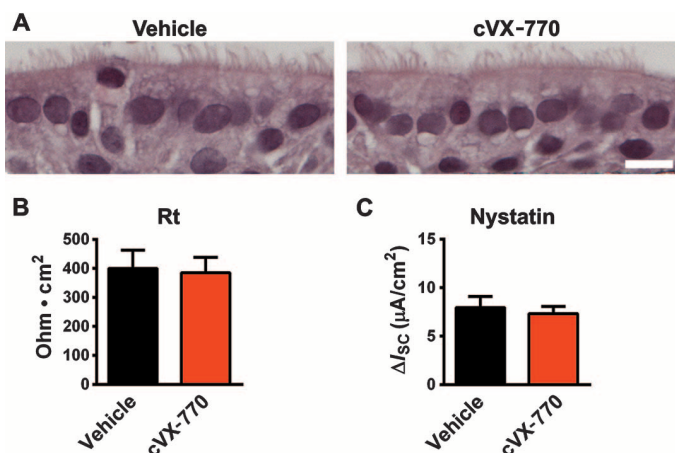


Fig. 6. Key physiological properties were not altered in chronically VX-770-treated HBE cultures. (A) Microscopy after hematoxylin and eosin (H&E) staining of HBE cultures did not reveal a detectable difference between VX-770- and vehicle-treated cells. Scale bar, 10 μ m. (B) Transepithelial resistance (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 hours, 5 μ M). Nystatin was added to the apical side in Ussing chambers. Primary HBE cultures were derived from five different individuals, and two to four replicates per individual were performed.

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 wild-type (ideal stability) or VX-809-rescued Δ F508 CFTR (low stability) by VX-770 bound to the CFTR molecule resulted in decreased wild-type CFTR function and absent Δ F508 CFTR function. These considerations reveal that chemical correction of low-stability CFTR mutants is complicated and necessitates precision.

Some airway diseases such as 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 wild-type CFTR function contrasts with our finding that chronic treatment with VX-770 reduced wild-type CFTR function. In addition, forskolin-stimulated I_{SC} in normal 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 wild-type 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 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 Δ F508 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 median effective concentration (EC_{50}) for acute application of VX-770 differs remarkably in G551D/ Δ F508 (EC_{50} , 236 ± 200 nM) and Δ F508/ Δ F508 (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 that 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 Δ F508 CFTR correction and turnover are needed.

After chronic VX-770 treatment, we also observed diminished Cl^- secretory responses to additions of the P2Y_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. Whereas the effects in CF cells can be explained by the restoration of CFTR inhibiting activity (36), the inhibition of ENaC-mediated Na^+ absorption in normal 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, which are crucial parameters to consider when extrapolating our data to the clinic, may also differ in vitro and in vivo. We therefore selected in vitro doses that mimicked clinically measured drug concentrations. For example, 5-day VX-770 treatments with 150 or 450 mg (administered as one dose per day) in patients resulted in VX-770 concentrations in blood plasma of 1.4 and 5.5 $\mu\text{g}/\text{ml}$, respectively (67), which are equivalent to ~ 3.5 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 Δ F508 in CF airways in vivo to alleviate symptoms of CF, potentiation of the small amount of corrected Δ F508 CFTR is

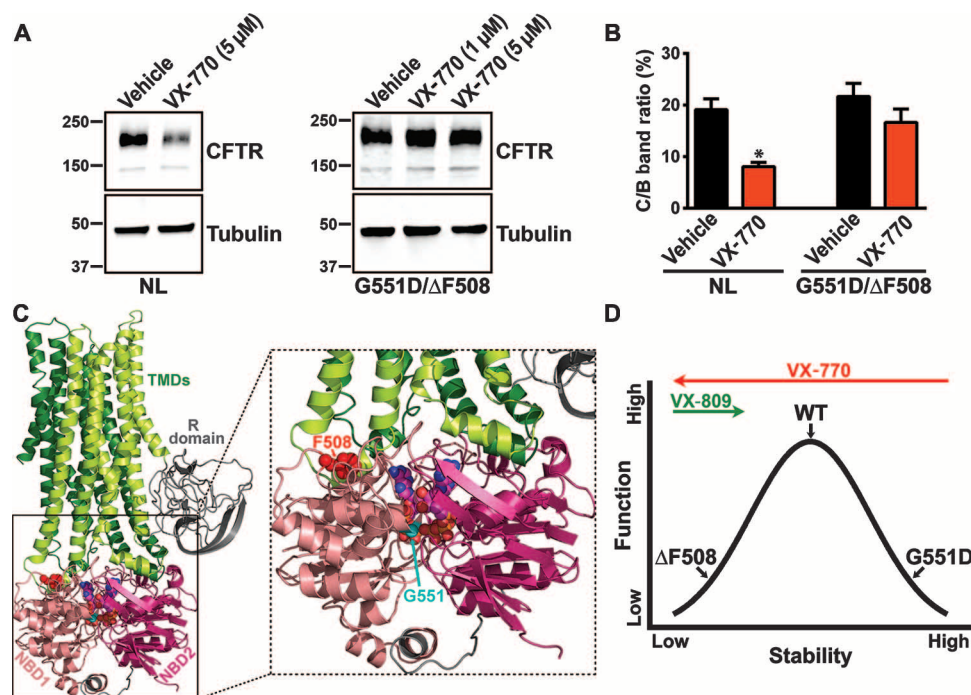


Fig. 7. VX-770 reduces stability of CFTR. (A) The amount of mature CFTR was reduced when normal HBE cells were chronically treated with VX-770 (48 hours, 5 μ M). G551D is more stable than normal CFTR, and the amount of mature G551D protein in CF cultures (G551D/ Δ F508) was not significantly reduced by 48-hour treatment with 5 μ M VX-770. (B) Quantification of C/B band ratio with chronic treatment of VX-770 at 5 μ M. C/B band ratio was significantly decreased in normal cells chronically treated for 48 hours with 5 μ M VX-770 (* P = 0.008) (n = 3, cultures from three different normal individuals). The reduction of C/B band ratio in G551D/ Δ F508 cells chronically treated for 48 hours with 5 μ M VX-770 was not statistically significant [n = 3, cultures from three different CF patients (G551D/ Δ F508)]. (C) Structural model showing positions of G551D and F508 in the CFTR molecule. TMDs, transmembrane domains. (D) Illustration representing the proposed relationship between function and stability of CFTR variants. Wild-type (WT) CFTR has an intermediate stability that allows for optimal function. G551D CFTR is a more rigid protein that exhibits increased stability compared to WT 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 WT CFTR. However, VX-770 causes destabilization of WT CFTR and VX-809-corrected Δ F508 CFTR, diminishing their function. VX-809 increases the stability of Δ F508, bringing it closer to resembling WT CFTR, but this increased stability is diminished when VX-770 is present.

required. However, combination approaches to restore Δ F508 CFTR function in CF to date have not considered drug-drug interactions of clinically relevant coadministered modulator compounds. On the basis of our study and the 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 Δ F508 mutation. Furthermore, understanding the impact that potentiator compounds, such as VX-770, have on the stability of apical wild-type 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-molecule 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 hours) with these compounds in primary HBE cells. Primary HBE cells from normal 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 I_{SC} (65, 71, 72). We examined differentiated primary CF HBE cells from at least three 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 BHK-21 cells stably expressing Δ F508 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 (UNC) Medical School Institutional Review Board. Primary normal 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% CO₂ for 3 to 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% CO₂. BHK-21 cells stably expressing Extope- Δ F508 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 to 20% gradient SDS-polyacrylamide gel electrophoresis gels (Bio-Rad) and then transferred to nitrocellulose. Blots were probed with mouse monoclonal anti-CFTR antibodies and then with IRDye 680-goat anti-mouse immunoglobulin G (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 Δ F508 CFTR

BHK-21 cells expressing Extope- Δ F508 CFTR were pretreated with compounds (VX-809, 5 μ M; VX-770, 5 μ M) for 24 hours 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 phosphate-buffered saline and fixed in 10% neutral buffered formalin before being embedded in paraffin and H&E-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 (Physiologic Instruments), HBE cultures were equilibrated to 37°C in a bilateral bath of Krebs bicarbonate–Ringer buffer [KBR; 140 mM Na⁺, 120 mM Cl[−], 5.2 mM K⁺, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 2.4 mM HPO₄^{2−}, 0.4 mM H₂PO₄[−], 25 mM HCO₃[−], and 5 mM glucose (pH 7.4)] and circulated with 95% O₂–5% CO₂. I_{SC} were measured as previously described (65, 71, 72). All normal HBE cultures were measured in bilateral KBR. Unless noted otherwise, CF HBE cultures were measured with high-potassium, low-chloride 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 & Analyze (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

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 mass/charge ratio (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 min, respectively. To quantify drug concentrations in epithelial cells, cell lysates were extracted twice with equal volume of methyl *tert*-butyl ether (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 and extracted, and 5 µl was 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 $\Delta\Delta G$ of mutation for G551D mutant CFTR using the Eris suite as described previously (43, 44). Eris algorithms repack 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 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 MATERIALS

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- Fig. S1. Chronic VX-770 treatment alters responses to amiloride and UTP in CF HBE cells.
- Fig. S2. Chronic VX-770 treatment inhibits functional rescue of $\Delta F508$ by VX-661.
- Fig. S3. Chronic VX-770 treatment alters responses to amiloride and UTP in normal HBE cells.
- Fig. S4. VX-770 and VX-809 concentrations were measured in treated HBE cells.
- Fig. S5. G551D mutation in the NBD1 stabilizes CFTR protein.
- Table S1. VX-770 treatment restores G551D function.
- Table S2. Chronic VX-770 treatment alters responses to amiloride and UTP.
- Table S3. Chronic VX-770 treatment inhibits functional rescue of $\Delta F508$ by VX-809.
- Table S4. Chronic VX-770 treatment inhibits functional rescue of $\Delta F508$ by VX-661.
- Table S5. VX-770 diminishes biochemical correction by increasing turnover of corrected $\Delta F508$ CFTR.
- Table S6. VX-770-induced hindrance of $\Delta F508$ correction is dose-dependent.
- Table S7. VX-770 at low dose (50 nM) affects I_{SC} of VX-809-corrected CF HBE cells ($\Delta F508/\Delta F508$).
- Table S8. VX-770 affects C/B band ratio in CF HBE ($\Delta F508/\Delta F508$).
- Table S9. Chronic VX-770 treatment decreases the function of wild-type CFTR.
- Table S10. Transepithelial resistance and nystatin responses were not altered in HBE cultures chronically treated with VX-770.
- Table S11. VX-770 and VX-809 concentrations were measured in HBE cell lysates.
- Table S12. VX-770 reduces stability of CFTR.

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