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.
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, 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 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 HCO3− transport by secretory and absorptive epithelial cells in multiple organs, including lungs, pancreas, liver, and intestine. In the lungs, 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 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 ΔF508 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 ΔF508 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 ΔF508 CF patient population is to promote transfer of the endoplasmic reticulum (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 ΔF508 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 ΔF508 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 ΔF508 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 (ΔF508/ΔF508) 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 ΔF508 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 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 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 hours with VX-770 or vehicle in the basolateral medium, and then transepithelial short-circuit currents (Isc) 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 CFTRinh−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 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 cultures (fig. S1B). The average uridine triphosphate (UTP) responsiveness, an index of Ca2+-activated Cl− channel (CaCC) activity, was reduced with chronic as compared to acute VX-770 administration (fig. S1C and table S2).

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**Fig. 1. VX-770 treatment restores G551D function.** Electrophysiological properties of G551D/ΔF508 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 Isc 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 VX-770, *P = 0.0054) (C), forskolin + aVX-770 (D), and CFTRinh−172 (E). Primary CF HBE cultures (G551D/ΔF508) were derived from three different patients, and three to four replicates were performed per patient for a total of 10 measurements per treatment.
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 hours, and then Ussing chamber experiments were performed to measure ΔF508 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 cultures (Fig. 2, A and 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. 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 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 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 CFTRinh-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 ΔF508 correction in CF HBE cultures (ΔF508/ΔF508) 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 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 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 ΔF508 CFTR was diminished, and instead, the

Fig. 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 hours at 5 μM each. (B) Δ$I_{SC}$ 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). Primary CF HBE cells treated with VX-809 responded to acute VX-770 exposure ($P = 0.0177$, 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 VX-809 + VX-770). (D) The response to CFTRinh-172 observed in VX-809–treated cells ($P = 0.0209$, VX-809 versus vehicle) was significantly abrogated in VX-809 + VX-770–treated cells ($P = 0.0031$, VX-809 versus VX-809 + VX-770). Primary HBE cultures (ΔF508/ΔF508) were derived from six different patients, and two to four replicates were performed per patient for a total of 15 measurements per condition.
The amount of remaining mature 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 hours 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 hours. 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 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 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. 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 ΔF508 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 CFTRinh-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...
vehicle- or VX-770–treated HBE cultures (Fig. 6C and table S10), suggesting intact Na⁺,K⁺-ATPase activity (vehicle- or VX-770 or 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 CFTRinh-172 (yields average ΔSc (μA/cm²)) was observed in cells 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 (ΔF508/ΔF508), the presence of chronic VX-770 at 50 nM increased 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 two 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 (ΔF508/ΔF508). 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 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 (ΔF508/ΔF508) from four different patients were analyzed. (E) Representative Western blot of CF HBE cells (ΔF508/ΔF508) showing decrease of VX-809–corrected ΔF508 as chronic VX-770 concentrations were increased.

**Fig. 4. VX-770–induced hindrance of ΔF508 correction is dose-dependent.** (A) Isc traces of HBE cells (ΔF508/ΔF508) recorded in using 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 CFTRinh-172 (yields average ΔSc (μA/cm²)) was observed in cells 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 (ΔF508/ΔF508), the presence of chronic VX-770 at 50 nM increased 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 two 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 (ΔF508/ΔF508). 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 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 (ΔF508/ΔF508) from four different patients were analyzed. (E) Representative Western blot of CF HBE cells (ΔF508/ΔF508) showing decrease of VX-809–corrected ΔF508 as chronic VX-770 concentrations were increased.

**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 markedly reduced the amount of mature CFTR in both normal 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 normal cultures (Fig. 7B, NL, and 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 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 ΔF508 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 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 as a logical agent to treat CF patients with at least one copy of the G551D mutation.
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 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/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 about 15% of normal function in ΔF508/ΔF508 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 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 coadministration 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 treatment 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). 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 wild-type or ΔF508 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
between VX-770 (H&E) staining of HBE cultures did not reveal a detectable difference between VX-770- and vehicle-treated cells. Scale bar, 10 μm. (B) Trans epithelial 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 μM). 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.

An 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 ΔF508 CFTR (low stability) by VX-770 bound to the CFTR molecule resulted in decreased wild-type 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 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 jsc 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 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 median effective concentration (EC50) for acute application of VX-770 differs remarkably in G551D/ΔF508 (EC50, 236 ± 200 nM) and ΔF508/ΔF508 (EC50, 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 ΔF508 CFTR correction and turnover are needed.

After chronic VX-770 treatment, we also observed diminished Cl− secretory responses to additions of the P2Y2 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 μg/ml, respectively (67), which are equivalent to ~3.5 and ~14 μM. Current clinical trial 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 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 ΔF508 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 ΔF508. 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 ΔVOC (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 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 (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-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 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 ΔF508 CFTR**

BHK-21 cells expressing Extope-ΔF508 CFTR were pretreated with compounds (VX-809, 5 μM; VX-770, 5 μM) for 24 hours at 37°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 DMRB Inverted Microscope with a 40x 1.0 numerical aperture oil objective.

Isc 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 HPO42−, 0.4 mM H2PO4−, 25 mM HCO3−, and 5 mM glucose (pH 7.4)] and circulated with 95% O2–5% CO2. Isc 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 CFTRinh-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). Isc traces were imported to and processed in Origin 9.0 (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 reconstituted 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 ΔG 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
www.sciencetranslationalmedicine.org/cgi/content/full/6/246/246ra96/DC1

Fig. S1. HBE cultures do not respond to VX-770. HBE cultures were not subjected to VX-770 treatment.

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 ΔF508 by VX-809.
Table S4. Chronic VX-770 treatment inhibits functional rescue of ΔF508 by VX-809.
Table S5. VX-770 diminishes biochemical correction by increasing turnover of corrected ΔF508.
Table S6. VX-770-induced hindrance of ΔF508 correction is dose-dependent.
Table S7. VX-770 at low dose (50 nM) affects ISc of VX-809–corrected CF HBE cells (ΔF508/ΔF508).
Table S8. VX-770 affects C/B band ratio in CF HBE (ΔF508/ΔF508).
Table S9. Chronic VX-770 treatment reduces 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.

REFERENCES AND NOTES


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