SLAS Discovery
2018, Vol. 23(2) 111–121

DOI: 10.1177/2472555217729790
journals.sagepub.com/home/jbx
SAGE

Use of a High-Throughput Phenotypic Screening Strategy to Identify Amplifiers, a Novel Pharmacological Class of Small Molecules That Exhibit Functional Synergy with Potentiators and Correctors

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Abstract

Cystic fibrosis (CF) is a lethal genetic disorder caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Despite recent groundbreaking approval of genotype-specific small-molecule drugs, a significant portion of CF patients still lack effective therapeutic options that address the underlying cause of the disease. Through a phenotypic high-throughput screen of approximately 54,000 small molecules, we identified a novel class of CFTR modulators called amplifiers. The identified compound, the characteristics of which are represented here by PTI-CH, selectively increases the expression of immature CFTR protein across different CFTR mutations, including F508del-CFTR, by targeting the inefficiencies of early CFTR biosynthesis. PTI-CH also augments the activity of other CFTR modulators and was found to possess novel characteristics that distinguish it from CFTR potentiator and corrector moieties. The PTI-CH–mediated increase in F508del-CFTR did not elicit cytosolic or endoplasmic reticulum–associated cellular stress responses. Based on these data, amplifiers represent a promising new class of CFTR modulators for the treatment of CF that can be used synergistically with other CFTR modulators.

Keywords

amplifiers, high-throughput screening, CFTR modulator, PTI-CH, phenotypic assays

Introduction

Cystic fibrosis (CF) is an autosomal recessive, multisystem disease caused by defects in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which codes for an ion channel that mediates chloride transport across epithelial cell membranes.[1] CFTR mutations include those that reduce levels of *CFTR* messenger RNA (mRNA) or prevent biosynthesis of CFTR protein, as well as those that encode misfolded, unstable, gating-defective, chloride conductance-defective, or otherwise nonfunctional protein.[1,2]

Currently, two types of small-molecule CFTR modulators have been developed that target the underlying defective mutant CFTR protein: potentiators and correctors. To date, ivacaftor (VX-770), a potentiator, and lumacaftor/ivacaftor, a corrector/potentiator combination, are the only two approved mutation-specific drug options for CF patients. Ivacaftor is approved for the treatment of CF patients with the *G551D* gating defect mutation, which affects approximately 3% to 4% of the CF population, as well as a handful of additional mutations that cause a similar deficit either in gating or in chloride conductance.[3–8] The utility of ivacaftor by itself is therefore limited to select genotype-specific patients.[3–6]

The *F508del* mutation is present in approximately 90% of CF patients, and a beneficial response to the lumacaftor/ivacaftor combination was observed in patients homozygous for *F508del*, whereas no benefit was seen in compound heterozygous patients in which the second allele is not *F508del*.[7,9,10] The effectiveness of this combination in compound heterozygous patients may be limited by reduced

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Received May 8, 2017, and in revised form Jul 17, 2017, Accepted for publication Aug 9, 2017.

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mutant CFTR protein substrates.[9] It is possible that increasing the available mutant CFTR substrate may enhance therapeutic benefit. Effective future modulators will need to treat the full range of CFTR genotypes with the goal of providing a meaningful therapeutic benefit for all patients. CF mutations interfere with the quantity or function of CFTR protein biosynthesis, a process dictated by a complex cellular protein quality control system that reduces its efficiency.[2,11] Current CFTR modulators are only as effective as the number of available substrates produced during biosynthesis.[9] New strategies are therefore needed to address this underlying substrate deficiency.

In this study, we report the results of a high-throughput screen (HTS) aimed at identifying novel first-in-class small molecules that exhibit functional synergy in combination with ivacaftor and lumacaftor (VX-809) and do not necessarily function as potentiators or correctors themselves. One of the active series identified, represented by PTI-CH, was shown to possess novel characteristics relative to known modulators. This newly identified class of CFTR modulators, which we have termed *amplifiers*, may advance our understanding of CF biology and, more important, be a promising candidate as a therapy for patients with CF.

Methods

HTS

A phenotypic HTS was used to identify novel modulators of CFTR function (**Fig. 1**). An immortalized cystic fibrosis bronchial epithelial cell line, CFBE41o$^-$,[12,13] which stably expresses a previously described halide-sensitive (H128Q/I152L variant) yellow fluorescent protein (YFP) reporter,[14–16] was used. This line is also stably transfected with a construct exogenously expressing F508del-CFTR complementary DNA (cDNA) under the control of a cytomegalovirus (CMV) promoter.[13,16,17] We refer to this cell line as CFBE41-YFP. The assay involves the addition of sodium iodide to the cells and kinetically monitoring the fluorescence signal following iodide addition.[18,19] If functional CFTR is present at the plasma membrane, iodide influx is accelerated and will quench fluorescence of the halide-sensitive reporter protein. This allows quantitation of CFTR function based on the forskolin- and genistein-dependent fluorescent quenching in response to modulators of CFTR function. Compounds were considered hits if they provided F508del-CFTR–mediated YFP quenching at 25% or greater than the positive control.

CFBE41-YFP cell culture was scaled up in multilayer hyperflasks (cat. 10024; Corning, Corning, NY) inoculated at a density of 75,000,000 cells per hyperflask. MEM alpha (cat. 12571071; Thermo-fisher, Waltham, MA) was used to maintain the cells. For every 500 mL MEM alpha, the following components were added: 50 mL Gibco (Gaithersburg, MD) fetal bovine serum (FBS), 7.5 mL 50 mg/mL G418, 5 mL 10,000 µg/10,000 U/mL penicillin/streptomycin, and 100 µL of 10 mg/mL puromycin. After 3 days in the hyperflask, cells were seeded onto 384-well clear bottom assay plates (cat. 3962; BD Falcon, Tewksbury, MA). Cells were plated (12,500 cells per well) in 80 µL complete MEM alpha using a Titertek Multidrop dispenser (Titertek-Berthold, Pforzheim, Germany). Plates were incubated for 2 days at 37 °C and 5% CO_2 prior to treatment with compound.

On day 2, 384-well plates containing DMSO compound stocks (at a concentration of ~10 mM and stored at −20 °C) were thawed at room temperature. Control compounds (96 samples per microplate) were added to the top four empty rows using a Beckman-Coulter Biomek FX liquid handler (cat. 4101500; Beckman-Coulter, Brea, CA). The 96 samples of control "compounds" consisted of 24 wells each of DMSO only (negative control), 3 µM lumacaftor (positive control), 10 µM tezacaftor (VX-661)[21] (corrector mechanism secondary positive control), and 3 µM suberoylanilide hydroxamic acid (SAHA, proteostasis mechanism secondary positive control). The medium in the 384-well plates seeded 2 days prior was aspirated down from 80 µL to 25 µL/well with a 384-well aspirator (Tecan Falcon 25 Model PW384; Tecan, Morrisville, NC). The Biomek FX liquid handling program was used to treat cells with 25 µL of 2× compound at a final DMSO concentration of 0.2% and a final compound concentration of ~10 µM. Cells were then incubated for 24 h at 37 °C and 5% CO_2.

On day 3, the 384-well plates were aspirated to 6 µL/well. Using a Multidrop dispenser (Model 832; Titertek-Berthold), 95 µL modified Dulbecco's phosphate-buffered saline (DPBS) was added to each well. The washing step was repeated by aspirating to 6 µL per well and replacing 95 µL/well DPBS. The plates were again aspirated back down to 6 µL/well, and 25 µL of (1.25×) 12.5 µM forskolin (cat. F6886; Sigma-Aldrich, St. Louis, MO) and 62.5 µM genistein (cat. G6649; Sigma-Aldrich) in modified DPBS were added to a final concentration of 10 µM forskolin and 50 µM genistein. Plates were incubated at room temperature for 1 h. Before reading plates on a FDSS/µCell instrument (model A11529-01; Hamamatsu, Hamamatsu City, Japan), the internal Ligand A reservoir was filled with NaI buffer (0.7 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.1 mM $MgCl_2$, 145 mM NaI, 8.1 mM $NaHPO_4$, pH 7.4). Plates were read individually, collecting a 10-s baseline signal before injection with NaI buffer (30 µL delivered 4 mm above the bottom of the plate within 1 s), followed by a reading every 500 ms for 300 reads or a total of 2 min and 40 s. In between each plate, the in-instrument wash cycle was used to flush tips with water five times. After the reads were complete, data were analyzed to derive the YFP quenching rate the percent quench at 30 s after NaI addition as reporters for CFTR chloride channel activity.

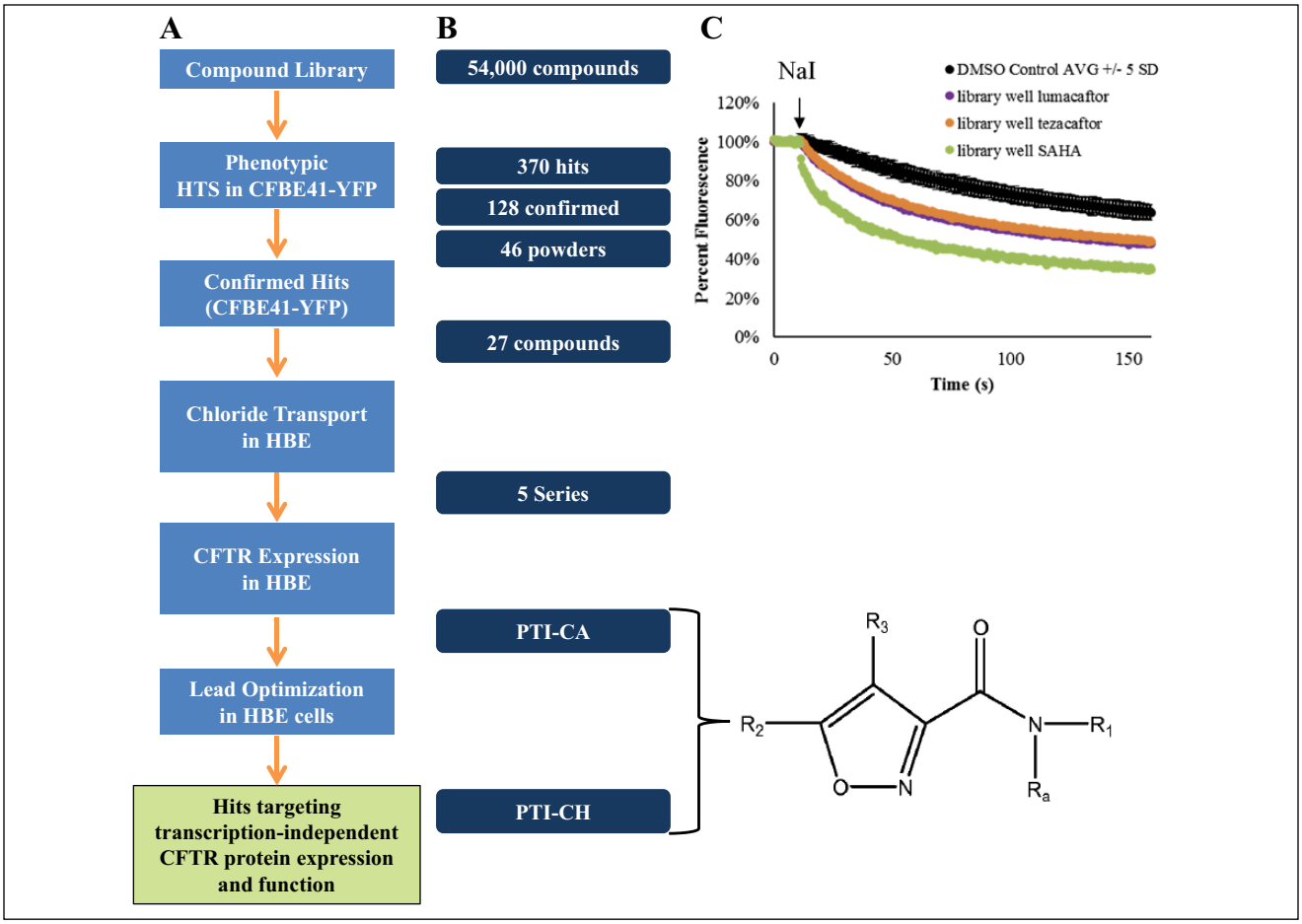

Figure 1. Screening strategy, compound triage, and potency and maximum efficacy improvements during optimization of PTI-CH. (**A**) The screening funnel showing the steps used to confirm and characterize compound hits from the small-molecule library. (**B**) The number of compounds at the beginning and end of each of the triage steps in the screening process. The common pharmacophore for PTI-CA and PTI-CH is shown, with R1-3 and Ra side groups as described.[20] (**C**) The yellow fluorescent protein (YFP) fluorescence quenching in response to sodium iodide (NaI) addition is shown for the DMSO control wells from one plate (±5 standard deviations) in comparison to the three indicated reference compounds that were included as test compounds in single wells in the compound library. These same reference compounds were also present on each plate in control wells (see text). CFTR, cystic fibrosis transmembrane conductance regulator; HBE, human bronchial epithelial; HTS, high-throughput screen.

Primary Human Bronchial Epithelial Cell Culturing and Differentiation

Human bronchial epithelial (HBE) cells from non-CF and CF donors of the indicated genotypes were provided by the Tissue Procurement and Cell Culture Core of the Cystic Fibrosis and Pulmonary Diseases Research and Treatment Center of the University of North Carolina Marsico Lung Institute (Chapel Hill, NC). HBEs were cultured essentially as described[22] and were differentiated in an air-liquid interface essentially as described.[23] HBEs were differentiated with medium replacement three times a week for a minimum of 4 weeks prior to electrophysiology (Ussing chamber) or other experiments.

Measuring CFTR Activity in Ussing Chambers

Primary HBEs with CF-causing mutations were differentiated for a minimum of 4 weeks in an air-liquid interface on SnapWell filter plates (cat. 3801; Corning Costar, Corning, NY) prior to Ussing measurements. Cells were apically mucus-washed for 30 min prior to treatment with compounds. The basolateral medium was removed and replaced with medium containing the compound of interest diluted to its final concentration from DMSO stocks. Treated cells were incubated at 37 °C and 5% CO_2 for 24 h. At the end of the treatment period, the cells on filters were transferred to the Ussing chamber and equilibrated for 20 min in assay buffer (Coon's F12 without sodium bicarbonate [cat. F6636; Sigma-Aldrich], 10 mM HEPES, pH 7.4). The short-circuit current (Isc) was measured in voltage clamp mode (V_{hold} = 0 mV), and the entire assay was conducted at 36.0 °C to 36.5 °C. Once the voltages had stabilized, the chambers were clamped and were recorded with readings every 10 s. Following baseline current stabilization, the following were added, and changes in the current and resistance of the cells were monitored: (1) 10 µM benzamil to the apical chamber

to inhibit the ENaC sodium channel,[24] (2) 10 μM forskolin to both chambers to activate CFTR by phosphorylation, (3) 1 μM ivacaftor to both chambers to potentiate CFTR channel opening, and (4) 20 μM CFTRinh-172 to the apical chamber to inhibit CFTR Cl^- conductance.[25] The inhibitable current (the current that is blocked by CFTRinh-172) is reported as the CFTR activity, and increased activity in response to compound beyond that observed in vehicle-treated samples is the improvement of F508del-CFTR function imparted by the compound tested.

Approach to Determining Synergy versus Additivity with Other CFTR Modulators

To evaluate whether the combination of PTI-CH with other modulators was additive or synergistic in the study, the DMSO CFTR activity was subtracted from the 3-μM PTI-CH CFTR activity, and this difference was then added to the "without PTI-CH" treatments for ivacaftor, lumacaftor, and the dual combination of ivacaftor + lumacaftor. This "expected" additivity was then compared to the observed activity for the respective treatments "with PTI-CH," and the lumacaftor and ivacaftor + lumacaftor combinations were significantly greater (by two-way analysis of variance [ANOVA]) than the activity predicted for an additive interaction.

CFTR Immunoblotting

Cells were apically mucus-washed for 30 min prior to treatment with compounds. The basolateral medium was removed and replaced with medium containing the compound of interest diluted to its final concentration from DMSO stocks. Treated cells were incubated at 37 °C and 5% CO_2 for 24 h. Cells were lysed and supernatants collected as follows: cells were washed with cold phosphate-buffered saline (PBS) three times and then lysed with 100 μL IP lysis buffer (cat. PI-87788; Thermo-Fisher Scientific, Grand Island, NY) containing protease inhibitors (cat. 11697498001, cOmplete tablets; Sigma-Aldrich). Lysates were placed on ice for 20 min and then centrifuged at 100,000 × *g* for 30 min at 4 °C. Supernatants were collected and protein assay performed using Lowry reagent from Bio-Rad (Hercules, CA). Protein samples (20–40 μg) were loaded onto a 3% to 8% Tris-Tricine gel (cat. 345-0129; Bio-Rad). After electrophoresis, proteins were transferred onto nitrocellulose membrane. The nitrocellulose membrane was then blocked in Tris-buffered saline + 0.1% Tween-20 (TBST) containing 20% goat serum for 1 h at room temperature with gentle agitation. Primary CFTR antibody (anti-CFTR; UNC-596) was prepared at a 1:1000 dilution in TBST, added to the membrane, and incubated overnight with gentle agitation at 4 °C. The following day, the membrane was washed three times (5 min each) with TBST at room temperature with gentle agitation and then incubated with goat anti-mouse IgG horseradish peroxidase (HRP)–labeled secondary antibody (sc-2005; Santa Cruz, Santa Cruz, CA) at a 1:10,000 dilution in TBST containing 10% goat serum for 1 to 3 h at room temperature with gentle agitation. Subsequently, the membrane was washed three times (5 min each) in TBST at room temperature with gentle agitation, and SuperSignal Femto solution (cat. PI-34096; Thermo-Fisher Scientific) was used to develop the HRP signal. Following detection, the membrane was stripped (cat. 21059, Restore Western Blot Stripping Buffer; Thermo-Fisher Scientific), blocked, and incubated with anti–Na+/K+ ATPase antibody as a loading control (cat. 3010S; Cell Signaling Technologies, Danvers, MA) at a 1:2000 dilution overnight at 4 °C with gentle agitation. Pierce ECL 2 plus substrate (cat. PI80196; Thermo-Fisher Scientific) was used the following day to develop the HRP signal. Band B/C quantification of protein amount was performed using an AlphaImager, Protein Simple, San Jose, CA densitometry software.

Surface and Total Protein Expression

The CFTR Mutant Surface and Total Protein Expression Assay and the P-gp Mutant Surface and Total Protein Expression Assay (Sharp Edge Labs, Pittsburgh, PA) were used to evaluate the effects of compounds on surface and total protein levels of F508del-CFTR and G268V–P-gp, respectively, in HEK293 cells. The assays make use of a fluorogen-activating protein fused to F508del-CFTR or G268V–P-gp that is detected through the use of a dye, which only fluoresces when bound to the protein.[26] HEK293 cells stably transfected with constructs containing the peptide fused to F508del-CFTR or G268V–P-gp were incubated at 37 °C for 24 h with PTI-CH or one of two corrector compounds (VRT-325 or lumacaftor). Flow cytometry was then used to quantitate the levels of surface and total F508del-CFTR or G268V–P-gp protein using this system. A cell-impermeable dye was used to detect surface protein, while a cell-permeable dye detected total protein. Propidium iodide–positive cells were excluded from quantification.

RNA Isolation

Total RNA was isolated from nasal epithelial cells using the RNeasy Plus Universal Mini kit (cat. 73404; Qiagen, Oslo, Norway). Nasal epithelial lysate was thawed at room temperature. The lysate was agitated by running the Eppendorf tube across the top of a plastic Eppendorf tube rack twice. Samples were vortexed briefly and pipetted up and down five times with a P1000 pipette. Tubes were spun briefly in a benchtop centrifuge to allow the lysate to settle. The liquid was then transferred to a clean RNase-free Eppendorf tube, leaving behind the brush. An equivalent volume (about 350 μL) of 70% ethanol was added to the lysate, and the sample was mixed by pipetting up and down 7 to 10 times and vortexed briefly. The entire mixture was transferred, including all precipitate, to an RNeasy spin column in a 2-mL collection tube.

The sample was centrifuged for 1 min at 10,000 × *g* in a benchtop centrifuge, and the flow-through was discarded; this step was repeated for the remainder of the sample, if necessary. The column filter was washed by adding 700 µL RWT wash buffer to the RNeasy spin column and spinning for 30 s at 10,000 × *g*. Buffer RPE (500 µL) was then added to the RNeasy spin column, the sample was centrifuged for 30 s at 10,000 × *g*, and flow-through was discarded. Then, 500 µL Buffer RPE was added to the RNeasy spin column, and the sample was centrifuged for 2 min at 10,000 × *g*. The RNeasy spin column was then transferred to a new collection tube and centrifuged for 1 min at 16,000 × *g* to dry the membrane. The RNeasy spin column was then placed in a new 1.5-mL RNase-free Eppendorf tube, and 35 µL of 37 °C RNase-free water was added directly to the spin column membrane and allowed to incubate for 5 min at room temperature. The tube was centrifuged for 1 min at 16,000 × *g* to elute the RNA, and the RNA concentration was measured.

Results

A High-Throughput Phenotypic Screening Strategy Identifies a Novel CFTR Modulator

A highly curated library of approximately 54,000 small molecules selected for novelty and drug-like properties was screened via HTS using a phenotypic assay in CFBE41-YFP cells to detect F508del-CFTR function. The assay performance for the HTS was monitored by negative control wells and three different positive control compounds, present on every plate. The DMSO-only negative control had a coefficient of variance of 4%, and the average Z′ across the plates was 0.5 for SAHA and tezacaftor and 0.7 for lumacaftor. The screening strategy shown in **Figure 1A** was used to confirm and validate hits as follows. From the library, 370 primary hits were identified and retested to determine the concentration dependence of their activity (**Fig. 1B**). The three control compounds mentioned above were also added to their own single empty well position on a single plate within the library to see if they would be identified as a hit. **Figure 1C** shows the quenching data from the primary HTS for these "library well" reference compounds that were indeed identified as hits in the screen and went on to show a concentration dependence to their activity. These were excluded from further characterization.

Of the 128 confirmed compounds that showed a concentration-dependent response, 27 were selected for further testing of their ability to improve endogenously expressed CFTR electrophysiological function in HBE cells from patients homozygous for the *F508del* mutation (*F508del/F508del* HBE cells). Five series of compounds were identified with activity in both the *F508del* CFBE41-YFP cell line and in *F508del/F508del* HBE cells. Of the five series of compounds that showed activity in *F508del/F508del* HBE cells, one of the compounds, PTI-CA, was found to increase F508del-CFTR levels in these cells. Based on the drug-like properties of PTI-CA and its observed activity in HBE cells, we pursued an optimization campaign to improve the efficacy and potency of the series. Among the optimized compounds, PTI-CH was selected as a representative of the chemical class for further characterization. PTI-CH has superior potency and in vitro efficacy compared to its parent compound in both the *F508del* CFBE41-YFP cell line assay (**Fig. 2A**) and *F508del/F508del* HBE cells (**Fig. 2B**). While the relative potency and maximal effect for PTI-CH are greater than PTI-CA in both the cell line and the primary cells, approximately 10 times more compound is needed in the overexpression *F508del* CFBE41-YFP cell line assay than in the primary *F508del/F508del* HBE cells for both compounds. This difference may be due to the increased abundance in CFTR in the cell line system relative to the

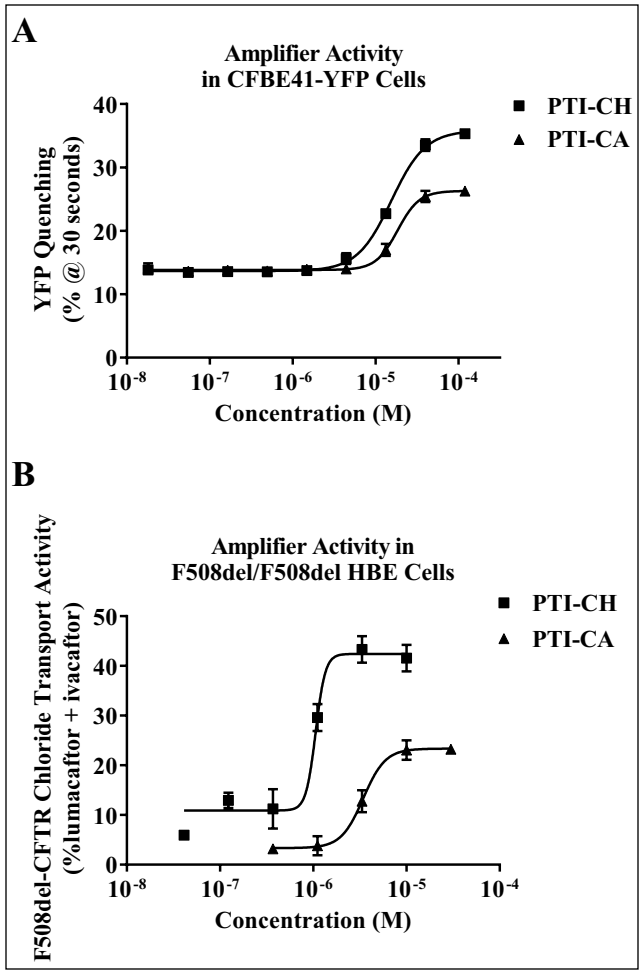

Figure 2. Potency and maximum efficacy improvements during optimization of PTI-CH. (**A**) Comparison of relative potency and activity of original hit compound PTI-CA with the optimized compound PTI-CH in the original CFBE41-YFP F508del–cystic fibrosis transmembrane conductance regulator (CFTR) functional assay. (**B**) Comparison of the relative potency and activity of PTI-CA with PTI-CH in the F508del/F508del human bronchial epithelial (HBE) CFTR functional assay. YFP, yellow fluorescent protein.

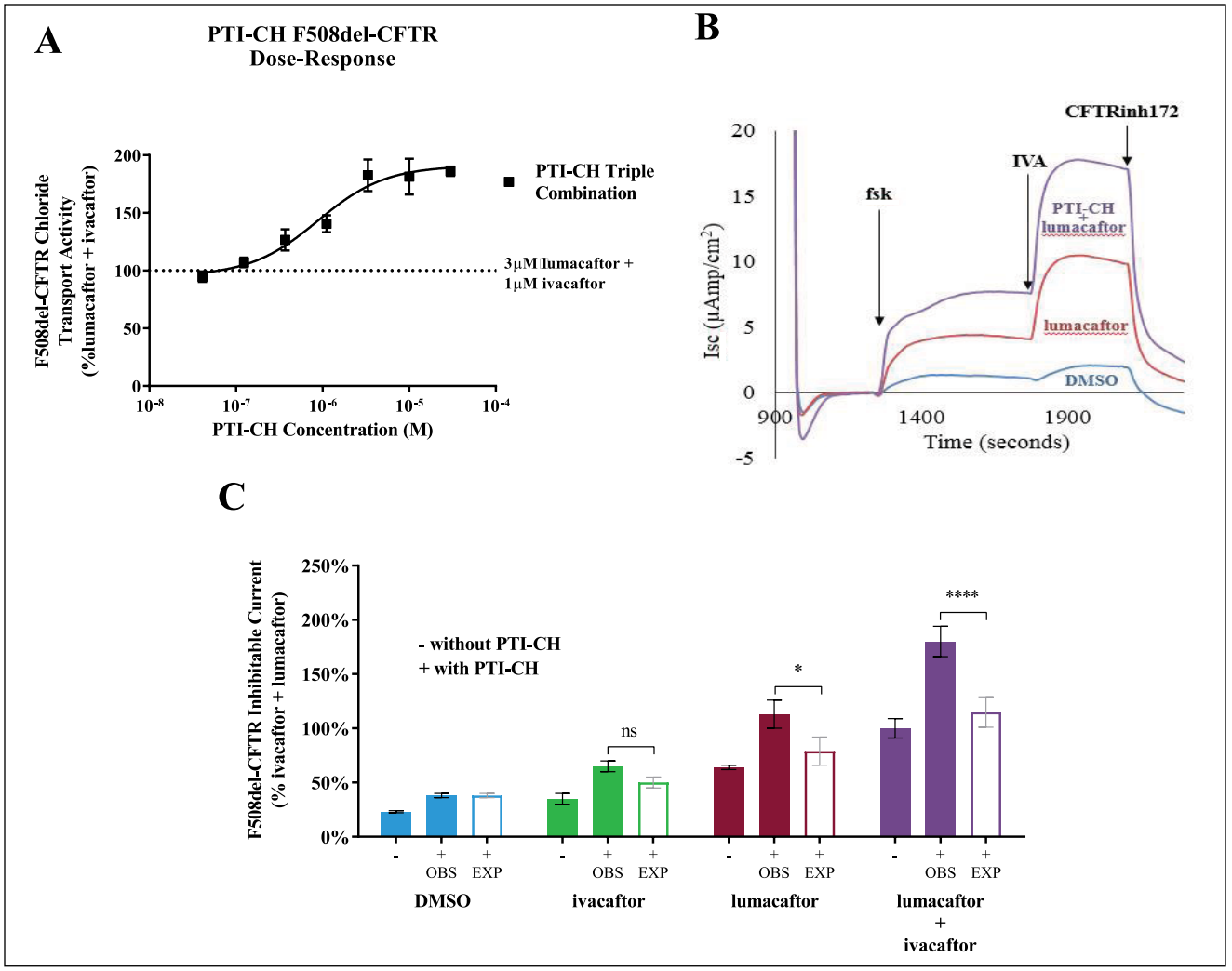

Figure 3. PTI-CH improves the functional rescue of F508del–cystic fibrosis transmembrane conductance regulator (CFTR) conferred by approved CFTR modulators. (**A**) PTI-CH shows a concentration-dependent increase in the activity of the combination of ivacaftor and lumacaftor in Ussing chamber short-circuit current measurements. (**B**) Representative Ussing chamber Isc traces are shown for the indicated treatments. Acute additions (10 μM forskolin, 1 μM ivacaftor, 20 μM CFTRinh172) during the Ussing assay are shown with arrows indicating the time of addition. All other compound incubations (3 μM lumacaftor with or without 3 μM PTI-CH) were for 24 h prior to the assay. Not shown is the 10 μM benzamil addition at the beginning of the assay to inhibit ENaC activity. (**C**) Summary of PTI-CH–mediated increases in F508del-CFTR CFTRinh172-sensitive current relative to other treatments. Concentrations as in panel **B**. Data are presented relative to the ivacaftor and lumacaftor combination. Solid bars represent means and black error bars represent the standard error of the mean (SEM) of three biological replicates. Open bars represent calculated values for expected additivity of PTI-CH with the indicated treatment, and gray error bars are drawn from the experimentally observed combinations. EXP, expected activity if PTI-CH were additive to the without PTI-CH condition; OBS, observed functional activity. See Methods for details. *ns*, non-significant. *$p < 0.05$, ****$p < 0.0001$ by two-way ANOVA with Tukey's multiple comparisons test.

endogenously expressing primary cells. Potency and maximal effective concentration differences between an overexpression cell line and *F508del/F508del* HBE cells have also been shown for lumacaftor.[27]

PTI-CH Increases F508del-CFTR Function in Primary HBE Cells and Augments the Activity of Other CFTR Modulators

Electrophysiological analysis was used to evaluate CFTR function in response to PTI-CH in comparison to and in combination with the CFTR modulators ivacaftor and lumacaftor in *F508del/F508del* HBE cells. The Ussing chamber was used to detect the short-circuit current across HBE monolayers, with the difference in short-circuit currents upon treatment with various compounds being measured to indicate their activity. The concentration-dependent activity of PTI-CH alone in HBE cells was recapitulated when incubated in combination with the corrector lumacaftor and acutely potentiated with ivacaftor (**Fig. 3A**). The forskolin- and ivacaftor-induced increases in the short-circuit current seen in vehicle control-treated cells were enhanced in cells treated with lumacaftor (**Fig. 3B**). Coadministration of PTI-CH with lumacaftor

further enhanced the levels of both the forskolin-responsive and ivacaftor-potentiated short-circuit currents, nearly doubling the current in response to both molecules during the assay. As shown in **Figure 3C**, measurement of the CFTRinh172-sensitive current demonstrated a 1.5- to 2-fold increase in F508del-CFTR function upon treatment with PTI-CH alone, in combination with ivacaftor, in combination with lumacaftor, and in combination with both lumacaftor and ivacaftor. The synergy of the combination of all three CFTR modulators provided the greatest activity, indicating that all three molecules act nonredundantly to provide increased CFTR chloride transport activity.

The results shown in **Figure 3** indicate that the addition of PTI-CH nearly doubles the activity of a corrector (lumacaftor) and potentiator (ivacaftor) in combination when each compound is used at a concentration greater than its EC_{90}.[27,28] Although the observed synergy, in particular in the triple combination, suggests that PTI-CH functions through a mechanism different from that of lumacaftor or ivacaftor, the possibility remained that PTI-CH functions as a corrector or potentiator with a distinct and complementary mechanism.

PTI-CH Is Neither a Potentiator nor a Corrector of CFTR but Possesses Novel Characteristics

To investigate whether PTI-CH acts as a potentiator, we assessed its ability to acutely increase forskolin-dependent F508del-CFTR activity in the Ussing chamber assay (**Fig. 4A**,**B**). In contrast to ivacaftor, PTI-CH induced no effect on CFTR current upon acute addition, consistent with a lack of potentiator activity (**Fig. 4A**). **Figure 4B** contains the summary of the mean responses to the acutely added CFTR modulators shown in the **Figure 4A** traces.

To determine whether PTI-CH acts as a corrector, we next examined its effect on F508del-CFTR maturation (**Fig. 4C**). The degree of CFTR maturation was determined by immunoblot analysis, measuring the conversion of the lower molecular weight, immature, core-glycosylated form of F508del-CFTR (the 150-kDa band, band B) into the higher molecular weight, mature, fully glycosylated form (the 250-kDa band, band C).[29,30] Treatment of *F508del/F508del* HBE cells with lumacaftor increased only the levels of the slower-migrating mature form of F508del-CFTR (**Fig. 4C**), consistent with its previously described activity as a corrector.[27] In contrast, PTI-CH increased both the immature and mature forms of F508del-CFTR, consistent with an increase in total CFTR protein and consistent with PTI-CH acting through a mechanism distinct from direct F508del-CFTR correction. Interestingly, the combined treatment with PTI-CH and lumacaftor led to an enhanced increase in F508del-CFTR protein greater than that observed with either modulator alone, underscoring the synergistic effects observed in the functional assay (see **Fig. 3**).

HEK293 cells stably transfected with reporter constructs for F508del-CFTR or a related mutant ATP-binding cassette (ABC) transporter protein, G268V–P-gp, fused to a fluorogen-activating protein, were subjected to flow cytometry to quantitate cell surface and total protein levels. The G268V–P-gp was used as a control for specificity. The effect of PTI-CH compared with two distinct and established correctors, lumacaftor (previously shown to be selective for F508del-CFTR correction[27]) and VRT-325 (previously shown to also be highly active in correcting G268V–P-gp[27]), is shown in **Figure 4D**. While both correctors caused a modest increase in the cell surface levels of F508del-CFTR and a decrease in total protein, PTI-CH increased total F508del-CFTR levels to nearly 300% of vehicle-treated cells. This effect was selective, as PTI-CH did not increase total G268V–P-gp levels, indicating that PTI-CH does not increase the levels of other mutant membrane proteins and likely has a mechanism of action with some specificity toward CFTR. VRT-325 produced a 400% increase in surface expression of G268V–P-gp, with no increase in the total protein expressed (**Fig. 4D**).

The increase in the levels of total F508del-CFTR protein in response to PTI-CH could be explained by increasing the levels of CFTR mRNA. Primary bronchial epithelial cells were treated for 24 h with PTI-CH, and RNA was isolated and subjected to quantitative reverse transcription (RT)–PCR to measure the levels of CFTR mRNA. **Figure 4E** shows that PTI-CH indeed increased CFTR mRNA levels by ~1.5- to 2-fold in these cells, similar to the magnitude of its effect in functional assays (see **Fig. 3C**). Importantly, the increase in CFTR mRNA due to PTI-CH was not dependent on the F508del mutation, as an increase was seen in HBE cells derived from a donor with the F508del/F508del genotype, as well as in HBE cells from a non-CF donor (**Fig. 4E**). While this manuscript was in revision, a separate study was published that showed that PTI-CH acts posttranscriptionally to increase endogenously expressed CFTR that had been engineered to have another CF-causative mutation, c.3700 A>G.[31]

In addition, CFTR mRNA was increased in the CFBE41-YFP cells where CFTR cDNA was expressed under the control of an exogenous promoter (data not shown) and in the HEK293 surface expression reporter system (see **Fig. 4D**) where only the open reading frame (ORF) of *F508del-CFTR* was present (**Fig. 4F**). The same HEK293 reporter system expressing the *G268V–P-gp* ORF did not respond to PTI-CH. The ability of PTI-CH to selectively increase CFTR mRNA across different expression contexts is a novel aspect to its mechanism of action that will require further studies.

The PTI-CH–Mediated Increase in Misfolded CFTR Does Not Trigger a Cellular Stress Response

Since F508del-CFTR is a misfolded membrane protein, it was important to eliminate the possibility that an increase in expression of the mutant protein in response to PTI-CH triggers

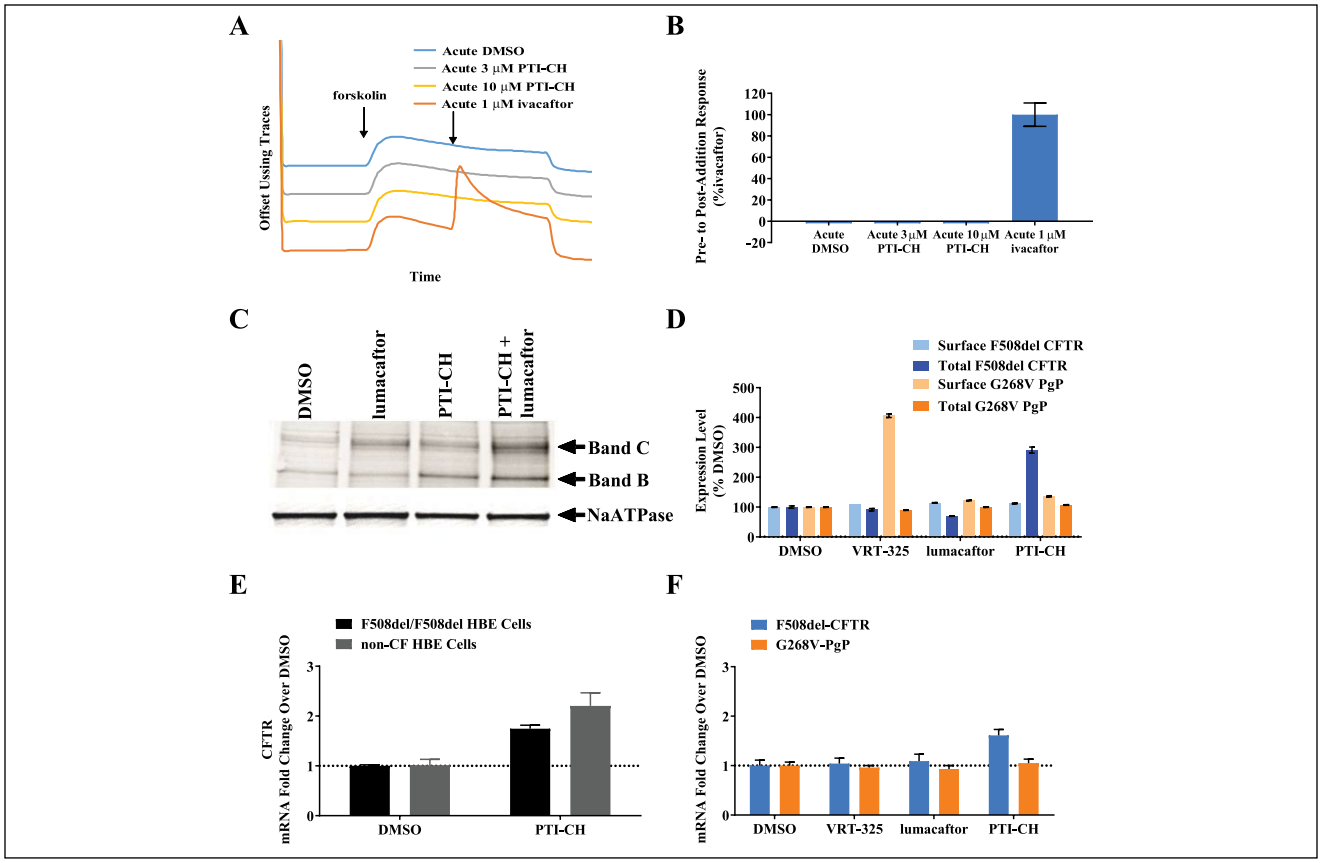

Figure 4. PTI-CH is distinct from the potentiator and corrector class of cystic fibrosis transmembrane conductance regulator (CFTR) modulators. (**A**) Average Isc traces of the indicated treatments are shown for *F508del/F508del* human bronchial epithelial (HBE) cells that had been incubated for 24 h with 3 μM lumacaftor. (**B**) Quantitation of the three chambers assayed for each treatment shown in panel **A** relative to the 1-μM ivacaftor acute addition. Bars represent means, and error bars represent the standard error of the mean (SEM) of three biological replicates. (**C**) Immunoblot of *F508del/F508del* HBE cells showing the levels of immature (band B) and mature (band C) of F508del-CFTR protein. NaATPase was used as a loading control. Lumacaftor and PTI-CH were both used at 3 μM, and F508del-CFTR was detected with MAB-596. (**D**) Surface and total expression of F508del-CFTR and G268V–P-gp were measured in the Sharp Edge Labs Mutant Surface and Total Protein Expression Assays in HEK293 cells. All compounds were used at 10 μM. Bars represent means, and error bars represent the SEM of six biological replicates. (**E**) CFTR messenger RNA (mRNA) levels were quantitated in *F508del/F508del* or non-CF HBE cells incubated for 24 h with 3 μM PTI-CH or DMSO prior to CFTR mRNA quantitation. Bars represent means, and error bars represent the SEM of three biological replicates. (**F**) F508del-CFTR and G268V–P-gp mRNA levels were measured from the Sharp Edge Labs stable transfectant HEK293 cells used in panel **D**. All compounds were used at 10 μM. Bars represent means, and error bars represent the SEM of four biological replicates.

cellular stress responses, such as the cytosolic heat shock response or the endoplasmic reticulum–based unfolded protein response (UPR) (**Fig. 5**). *F508del/F508del* HBE cells were treated either with PTI-CH, the CFTR potentiator ivacaftor, or an F508del-CFTR corrector, tezacaftor. Following 24 h of incubation with these modulators, gene expression of stress response constituents was measured using quantitative PCR (qPCR). For the cytosolic stress response, we selected genes that play induced or constitutive roles in this response: *HSPA1A* (Hsp70A1),[32] *HSPA8* (Hsc70),[33] *HSP90AA1* (Hsp90),[34] and *HSP90B1* (GRP94).[35] No increase in mRNA levels of these stress genes was detected in response to the PTI-CH–induced increase in F508del-CFTR expression (**Fig. 5A**). Possible activation of the UPR was assessed by measuring the transcript levels of the following genes, which are transcriptional targets of the three arms of the UPR signaling pathway: *HSPA5* (BiP),[36] *DNAJB9* (ERdj4),[37] *DNAJC3* (p58-IPK),[38] *EDEM*,[37] *ATF4*,[36] *DDIT3* (CHOP),[36,37] and both total and spliced *XBP1*.[36] In response to treatment with PTI-CH, none of the UPR genes exhibited an increase in transcript levels (**Fig. 5B**), indicating that the UPR is not activated by the PTI-CH–mediated increase in F508del-CFTR expression. The other two modulators, ivacaftor and tezacaftor, both resulted in increases in CHOP, suggesting those modulators activate some signaling through the UPR in vitro in primary HBE cells derived from *F508del/F508del* donors. In summary, an increase in F508del-CFTR expression in response to PTI-CH activated neither the cytosolic stress response nor the UPR,

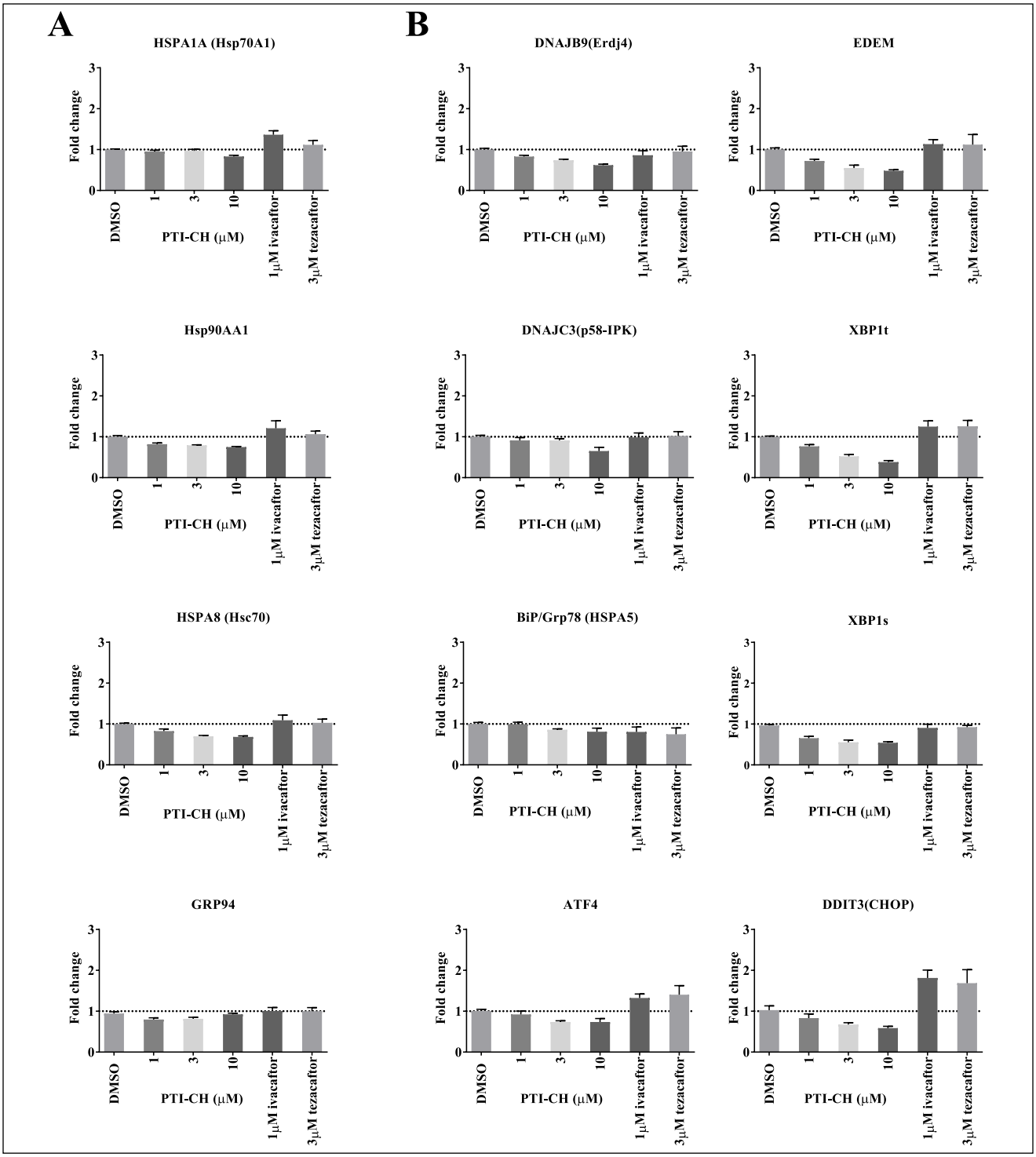

Figure 5. The increase in F508del–cystic fibrosis transmembrane conductance regulator (CFTR) does not result in the induction of cytosolic stress or endoplasmic reticulum (ER)–associated unfolded protein response pathways. (**A**) Quantitative PCR (qPCR) results showing the effect of incubation with PTI-CH for 24 h at the indicated concentrations on the indicated HSF1-regulated messenger RNAs (mRNAs) in *F508del/F508del* human bronchial epithelial (HBE) cells. (**B**) qPCR results showing the effect of incubation with PTI-CH for 24 h at the indicated concentrations on the indicated UPR-regulated mRNAs in *F508del/F508del* HBE cells. Bars represent means, and error bars represent the standard error of the mean (SEM) of three biological replicates.

consistent with this increase not leading to an induction of cellular stress.

Discussion

As CFTR is a complex, multidomain, membrane-spanning protein, it undergoes tightly regulated steps during its biosynthesis to achieve a correctly localized and functional mature protein within epithelial cells.[2] Mutations in CFTR affect the function or quantity of the CFTR channel at the cell surface.[1,2] Current CFTR modulators are only as effective as the amount of available substrate protein.[9] New strategies are therefore needed to address this underlying substrate limitation. While the recent regulatory approval of ivacaftor and lumacaftor/ivacaftor marks a major step forward in mutation-specific CFTR modulator-based therapies for CF,[3–6,9,10] there is still a significant unmet therapeutic need for patients with the disease. Compared with the efficacy achieved with ivacaftor for patients with the *G551D* mutation,[3,4] there is room for further benefit in lung function for patients with two copies of *F508del* for whom lumacaftor/ivacaftor is approved.[9,10] An even greater need remains for patients with only one copy of *F508del*, in which their reduced levels of F508del-CFTR protein may prevent them from realizing a clinical benefit with available therapies, owing to the lack of sufficient substrate F508del-CFTR protein for the corrector and potentiator.[9] Therapeutics that overcome the substrate limitation of F508del-CFTR, making F508del-CFTR available for other modulators to act upon, have the potential to address the unmet need for the most CF patients with one or two *F508del* alleles. In addition, if this activity is independent of the underlying CFTR mutation, these types of therapeutics might offer potential for all CF patients.

In this study, we report the results of an HTS campaign aimed at identifying novel pharmacological classes of small molecules that exhibit functional synergy with lumacaftor and ivacaftor. One of the active series identified, represented here by PTI-CH, augments the activity of other CFTR modulators and possesses novel characteristics relative to known modulators. This newly identified class of modulators functions neither as corrector nor as potentiator. We demonstrate that PTI-CH overcomes the inefficiencies of upstream CFTR biosynthesis by selectively increasing the expression of immature CFTR protein across different CFTR mutations, including F508del-CFTR.

No significant increases were detected among cellular stress response genes due to the amplifier-mediated increase in mutant F508del-CFTR expression. Interestingly, ivacaftor and tezacaftor both appear to induce some UPR signaling (see **Fig. 5**). However, the significance of these observations is unclear. PTI-CH appears to selectively increase the amount of CFTR protein available for other modulators to act upon and, thus, enhance the therapeutic benefit afforded by the currently approved corrector and potentiator classes of drugs. We have classified this novel type of small-molecule CFTR modulator as an amplifier.

While the screening strategy used to identify PTI-CH selected against direct transcriptional mechanisms, it did not preclude effects on CFTR biosynthesis and turnover at other points of regulation (e.g., by increasing transcript stability, reducing protein degradation, or improving translational efficiency). The mechanism by which PTI-CH selectively increases CFTR mRNA levels and protein expression is being intensely investigated and may provide further insight into how this novel modulator provides its benefit.

Acknowledgments

The authors thank the Tissue Procurement and Culturing Core at UNC, ChanTest, and Sharp Edge Labs for experimental support. We are grateful to Robert J. Bridges, Amita Thakerar, and Matthew Green from Chicago Medical School; Martin Mense, Hermann Bihler, and Eric Wong from the Cystic Fibrosis Foundation Therapeutics (CFFT); and William Skach from the Cystic Fibrosis Foundation (CFF) for their experimental help, expertise, insight, and helpful discussions. The authors also thank Brigid McEwan for technical support, Ken Longo for statistical analysis support, and Marija Zecevic for helpful comments on the manuscript. We acknowledge Caroline Ritchie for medical writing and F. Ulrich Hartl for editorial support.

Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors are employees of Proteostasis Therapeutics, Inc., and conducted this research as part of the company's drug discovery efforts. Proteostasis Therapeutics, Inc. has filed several patent applications relating to the amplifier class of CFTR modulators.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was funded in part by a Therapeutics Development Award from Cystic Fibrosis Foundation Therapeutics, Inc.

References

1. Egan, M. E. Genetics of Cystic Fibrosis: Clinical Implications. *Clin. Chest Med*. **2016**, *37*, 9–16.
2. Ong, T.; Ramsey, B. W. New Therapeutic Approaches to Modulate and Correct Cystic Fibrosis Transmembrane Conductance Regulator. *Pediatr. Clin. North Am*. **2016**, *63*, 751–764.
3. Ramsey, B. W.; Davies, J.; McElvaney, N. G.; et al. A CFTR Potentiator in Patients with Cystic Fibrosis and the G551D Mutation. *N. Engl. J. Med*. **2011**, *365*, 1663–1672.
4. Davies, J. C.; Wainwright, C. E.; Canny, G. J.; et al.; VX08-770-103 (ENVISION) Study Group. Efficacy and Safety of Ivacaftor in Patients Aged 6 to 11 Years with Cystic Fibrosis with a *G551D* Mutation. *Am. J. Respir. Crit. Care Med*. **2013**, *187*, 1219–1225.
5. De Boeck, K.; Munck, A.; Walker, S.; et al. Efficacy and Safety of Ivacaftor in Patients with Cystic Fibrosis and a Non-G551D Gating Mutation. *J. Cyst. Fibros*. **2014**, *13*, 674–680.

6. Moss, R. B.; Flume, P. A.; Elborn, J. S.; et al. Efficacy and Safety of Ivacaftor Treatment: Randomized Trial in Subjects with Cystic Fibrosis Who Have an *R117H-CFTR* Mutation. *Lancet Resp. Med*. **2015**, *32*, 524–533.
7. Cystic Fibrosis Foundation. *Patient Registry Annual Data Report 2015;* Cystic Fibrosis Foundation: Bethesda, Maryland, **2016**.
8. US Cystic Fibrosis Foundation, Johns Hopkins Medicine, The Hospital for Sick Children. Clinical and Functional Translation of CFTR. https://www.cftr2.org (accessed September 1, 2017).
9. Boyle, M. P.; Bell, S. C.; Konstan, M. W.; et al.; VX09-809-102 Study Group. A CFTR Corrector (Lumacaftor) and a CFTR Potentiator (Ivacaftor) for Treatment of Patients with Cystic Fibrosis Who Have a Phe508del CFTR Mutation: A Phase 2 Randomised Controlled Trial. *Lancet Respir. Med*. **2014**, *2*, 527–538.
10. Wainwright, C. E.; Elborn, J. S.; Ramsey, B. W.; et al. Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N. Engl. J. Med*. **2015**, *373*, 220–231.
11. Lukacs, G. L.; Mohamed, A.; Kartner, N.; et al. Conformational Maturation of CFTR but Not Its Mutant Counterpart (ΔF508) Occurs in the Endoplasmic Reticulum and Requires ATP. *EMBO J*. **1994**, *13*, 6076–6086.
12. Meng, Q. H.; Springall, D. R.; Bishop, A. E.; et al. Lack of Inducible Nitric Oxide Synthase in Bronchial Epithelium: A Possible Mechanism of Susceptibility to Infection in Cystic Fibrosis. *J Pathol*. **1998**, *184*, 323–331.
13. Bebok, Z.; Collawn, J. F.; Wakefield, J.; et al. Failure of cAMP Agonists to Activate Rescued ΔF508 CFTR in CFBE41o$^-$ Airway Epithelial Monolayers. *J. Physiol*. **2005**, *569*, 601–615.
14. Wachter, R. M.; Remington, S. J. Sensitivity of the Yellow Variant of Green Fluorescent Protein to Halides and Nitrate. *Curr. Biol*. **1999**, *9*, R628–R629.
15. Galietta, L. J.; Haggie, P. M.; Verkman, A. S. Green Fluorescent Protein–Based Halide Indicators with Improved Chloride and Iodide Affinities. *FEBS Lett*. **2001**, *499*, 220–224.
16. Sondo, E.; Tomati, V.; Caci, E.; et al. Rescue of the Mutant CFTR Chloride Channel by Pharmacological Correctors and Low Temperature Analyzed by Gene Expression Profiling. *Am. J. Physiol. Cell Physiol*. **2011**, *301*, C872–C885.
17. Vijftigschild, L. A. W.; van der Ent, C. K.; Beekman, J. M. A Novel Fluorescent Sensor for Measurement of CFTR Function by Flow Cytometry. *Cytometry Part A*.**2013**, *83A*, 576–584.
18. Lin, S.; Sui, J.; Cotard, S.; et al. Identification of Synergistic Combinations of F508del Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Modulators. *Assay Drug Dev. Technol*. **2010**, *8*, 669–684.
19. Sui, J.; Cotard, S.; Andersen, J.; et al. Optimization of a Yellow Fluorescent Protein–Based Iodide Influx High-Throughput Screening Assay for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Modulators. *Assay Drug Dev. Technol*. **2010**, *8*, 656–668.
20. Tait, B. D.; Cullen, M. D. (Proteostasis Therapeutics, Inc.). Methods of Modulating CFTR Activity. International Patent Appl. WO 2014/210159 A1. December 31, **2014**.
21. Pettit, R. S.; Fellner, C. CFTR Modulators for the Treatment of Cystic Fibrosis. *P. T*. **2014**, *39*, 500–511.
22. Fulcher, M. L.; Gabriel, S.; Burns, K. A.; et al. Well-Differentiated Human Airway Epithelial Cell Cultures. *Methods Mol. Med*. **2005**, *107*, 183–206.
23. Neuberger, T.; Burton, B.; Clark, H.; et al. Use of Primary Cultures of Human Bronchial Epithelial Cells Isolated from Cystic Fibrosis Patients for the Pre-clinical Testing of CFTR Modulators. *Methods Mol. Biol*. **2011**, *741*, 39–54.
24. Kleyman, T. R.; Cragoe, E. J., Jr. Amiloride and Its Analogs as Tools in the Study of Ion Transport. *J. Membr. Biol*. **1988**, *105*, 1–21.
25. Wang, X. F.; Reddy, M. M.; Quinton, P. M. Effects of a New Cystic Fibrosis Transmembrane Conductance Regulator Inhibitor on Cl^- Conductance in Human Sweat Ducts. *Exp. Physiol*. **2004**, *89*, 417–425.
26. Holleran, J.; Brown, D.; Fuhrman, M. H.; et al. Fluorogen-Activating Proteins as Biosensors of Cell-Surface Proteins in Living Cells. *Cytometry A* **2010**, *77*, 776–782.
27. Van Goor, F.; Hadida, S.; Grootenhuis, P. D. J.; et al. Correction of the F508del-CFTR Protein Processing Defect In Vitro by the Investigational Drug VX-809. *Proc. Natl. Acad. Sci. U.S.A*. **2011**, *108*, 18843–18848.
28. Van Goor, F.; Hadida, S.; Grootenhuis, P. D.; et al. Rescue of CF Airway Epithelial Cell Function In Vitro by a CFTR Potentiator, VX-770. *Proc. Natl. Acad. Sci. U.S.A*. **2009**, *106*, 18825–18830.
29. Chang, X-B.; Mengos, A.; Hou, Y-X.; et al. Role of N-Linked Oligosaccharides in the Biosynthetic Processing of the Cystic Fibrosis Membrane Conductance Regulator. *J. Cell Sci*. **2008**, *121*, 2814–2823.
30. Cheng, S. H.; Gregory, R. J.; Marshall, J.; et al. Defective Intracellular Transport and Processing of CFTR Is the Molecular Basis of Most Cystic Fibrosis. *Cell* **1990**, *63*, 827–834.
31. Molinski, S. V.; Ahmadi, S.; Ip, W.; et al. Orkambi® and Amplifier Co-Therapy Improves Function from a Rare CFTR Mutation in Gene-Edited Cells and Patient Tissue. *EMBO Mol. Med*. **2017** Jun 30. pii: e201607137. [Epub ahead of print].
32. Zhang, P.; Leu, J. I-J.; Murphy, M. E.; et al. Crystal Structure of the Stress-Inducible Human Heat Shock Protein 70 Substrate-Binding Domain in Complex with Peptide Substrate. *PLoS ONE* **2014**, *9*, e103518.
33. Stricher, F.; Macri, C.; Ruff, M.; et al. HSPA8/HSC70 Chaperone Protein: Structure, Function, and Chemical Targeting. *Autophagy* **2013**, *9*, 1937–1954.
34. Zuehlke, A. D.; Beebe, K.; Neckers, L.; et al. Regulation and Function of the Human *HSP90AA1* Gene. *Gene* **2015**, *570*, 8–16.
35. Gewirth, D. T. Paralog Specific Hsp90 Inhibitors—A Brief History and a Bright Future. *Curr. Top Med. Chem*. **2016**, *16*, 2779–2791.
36. Takayanagi, S.; Fukuda, R.; Takeuchi, Y.; et al. Gene Regulatory Network of Unfolded Protein Response Genes in Endoplasmic Reticulum Stress. *Cell Stress Chaperones* **2013**, *18*, 11–23.
37. Matsumura, K.; Sakai, C.; Kawakami, S.; et al. Inhibition of Cancer Cell Growth by GRP78 siRNA Lipoplex *via* Activation of Unfolded Protein Response. *Biol. Pharm. Bull*. **2014**, *37*, 648–653.
38. Rutkowski, D. T.; Kang, S-W.; Goodman, A. G.; et al. The Role of $p58^{IPK}$ in Protecting the Stressed Endoplasmic Reticulum. *Mol. Biol. Cell* **2007**, *18*, 3681–3691.