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TITLE:

POTENTIATORS OF DEFECTIVE ΔF508-CFTR CHANNEL GATING THAT DO NOT INTERFERE WITH CORRECTOR ACTION

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ΔF508-CFTR potentiators

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Abbreviations: CFBE, cystic fibrosis bronchial epithelial; CFTR, cystic fibrosis transmembrane conductance regulator; cyclopropanecarboxamide, 1-(2,2-difluoro-1,3-benzodioxol-5-yl)-N-[1-[(2R)-2,3-dihydroxypropyl]-6-fluoro-2-(2-hydroxy-1,1-dimethylethyl)-1H-indol-5-yl]; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; HA, human influenza hemagglutinin; HRP, horseradish peroxidase; VX-770, *N*-(2,4-di-*tert*-butyl-5-hydroxyphenyl) -4-oxo-1,4-dihydroquinoline -3-carboxamide; VX-809, 3-[6-[[[1-(2,2-difluoro-1,3-benzodioxol -5-yl)cyclopropyl]carbonyl] amino]-3-methyl-2-pyridinyl]-benzoic acid; VX-661, YFP, yellow fluorescence protein

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ABSTRACT

Combination drug therapies under development for cystic fibrosis (CF) caused by the ΔF508 mutation in CFTR include a 'corrector' to improve its cellular processing and a 'potentiator' to improve its chloride channel function. Recently, it was reported that the approved potentiator VX-770 (Ivacaftor) reduces ΔF508-CFTR cellular stability and the efficacy of investigational correctors, including VX-809 and VX-661, which might contribute to the modest reported efficacy of combination therapy in clinical trials. Here, we report the identification and characterization of potentiators that do not interfere with ΔF508-CFTR stability or corrector action. High-throughput screening and structure-activity analysis identified several classes of potentiators that do not impair corrector action, including tetrahydrobenzothiophenes, thiooxoaminothiazoles and pyrazole-pyrrole-isoxazoles. The most potent compounds have EC_{50} for ΔF508-CFTR potentiation down to 18 nM, and do not reduce corrector efficacy in heterologous ΔF508-CFTR expressing cells or in primary cultures of ΔF508/ΔF508 human bronchial epithelia. The ΔF508-CFTR potentiators also activated wildtype and G551D-CFTR, albeit weakly. The efficacy of combination therapy for CF caused by the ΔF508 mutation may be improved by replacement of VX-770 with a potentiator that does not interfere with corrector action.

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INTRODUCTION

Mutations in the cAMP-activated chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) cause the genetic disease cystic fibrosis (CF) in which defective epithelial fluid secretion and viscous mucus impairs the function of the lung, pancreas and other organs. The most common CFTR mutation, ΔF508, is present in at least one allele in ~ 90% of CF patients in North America. The ΔF508 mutation causes CFTR misfolding, with retention at the endoplasmic reticulum (ER), accelerated degradation, and impaired chloride channel function (Collins, 1992; Boucher, 2004; Davis, 2006; Riordan, 2008).

There has been great interest in development of small-molecule, CFTR-targeted therapeutics to treat the underlying CFTR defect responsible for the clinical manifestations in CF (Hanrahan et al., 2013; Rowe and Verkman, 2013; Ashlock and Olson, 2011). VX-770 (Ivacaftor) is approved for therapy of CF caused by mutations that affect CFTR gating but not cellular processing, including G551D and nine other point mutations (O'Reilley et al., 2013; Davis et al., 2012; Ramsey et al., 2011). A combination drug approach has been adopted for therapy of CF caused by the ΔF508-CFTR mutation – a 'corrector' to improve its cellular processing and a 'potentiator' to improve its chloride channel function. Two reported phase 3 clinical trials of combination therapy of the investigational corrector VX-809 with VX-770 showed modest clinical benefit in ΔF508 homozygous CF patients, with a range of 2.6-4 % improvement in lung function as measured by forced expiratory volume in 1 s (FEV_1) (NCT01807949; NCT01807923; Boyle et al., 2014; Vertex Pharmaceuticals press release, 2014). Two recent papers reported that VX-770 reduces ΔF508-CFTR cellular stability and the efficacy of VX-809 and a second investigational corrector, VX-661 (Veit et al., 2014; Cholon et al., 2014). The reduced corrector efficacy in cells chronically exposed to VX-770 results from impaired ΔF508-CFTR folding efficiency at the endoplasmic reticulum and accelerated plasma membrane turnover (Veit et al., 2014), which might account in part for the modest clinical efficacy of combination therapy.

We reasoned that a potentiator that does not reduce ΔF508-CFTR stability and corrector efficacy may be superior to VX-770 for combination therapy. The study here was done to identify potentiators that do not impair corrector action. This project was motivated by the observation that some

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potentiators, such as P5, do not destabilize ΔF508-CFTR or reduce corrector efficacy (Veit et al, 2014). The tetrahydrobenzothiophene P5 was the first reported CFTR potentiator, as identified by our lab using a cell-based functional high-throughput screen (Yang et al., 2003). Here, we screened 273 analogs of P5, as well as 60,000 unrelated drug-like synthetic small molecules, to identify potentiators that do not interfere with corrector action. Nanomolar-potency potentiators were identified and characterized that do not interfere with corrector action or impair ΔF508-CFTR cellular stability.

MATERIALS AND METHODS

Cell lines

Fisher rat thyroid **(**FRT) epithelial cells stably expressing ΔF508-, wildtype- or G551D-CFTR, together with halide-sensitive green fluorescent protein YFP-H148Q/I152L/F46L (Galietta et al., 2001) were as reported (Pedemonte et al., 2005; Ma et al., 2002). FRT cells were cultured in Coon's modified Ham's F12 medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For primary screening, FRT cells expressing ΔF508-CFTR were plated at 50,000 cells/well 18-24 h prior to screening.

CFBE41o- TetON (CFBE) cells expressing ΔF508-CFTR-HRP or ΔF508-CFTR-3HA were as described (Veit et al., 2012; Veit et al., 2014). CFBE cells were grown in minimal essential medium (MEM, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine and 10 mM HEPES. For propagation, cells were cultured in plastic flasks coated with 10 μg/ml human fibronectin, 30 μg/ml calf-skin collagen and 100 μg/ml bovine serum albumin diluted in LHC-8 basal medium (Invitrogen). For chemiluminescence assay, CFBE cells were plated in black, 96-well microplates (Costar, Corning Inc.) at 15,000 cells/well. ΔF508-CFTR-HRP expression was induced 24 h after plating with 0.5 μg/ml doxycycline for 2 d before assay.

Primary cultures of ΔF508/ΔF508 human bronchial epithelial cells

Primary human ΔF508/ΔF508 CF bronchial epithelial cells were isolated and then cultured on semi-permeable inserts (Snapwell; Corning, Lowell, MA) in ALI media at an air-liquid interface, as

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described (Yamaya et al., 1992; Fulcher and Randell, 2013). Media was changed every two to three days. All protocols involving the collection and use of human tissues and cells were reviewed and approved by the UCSF Institutional Review Board.

Compounds

60,000 chemically diverse, drug-like synthetic compounds (>90% with molecular size 250-500 Da; ChemDiv Inc., San Diego, CA) were used for primary screening. For optimization, 387 commercially available analogs of two chemical classes were tested, as well as 273 analogs of P5. Analogs of active compounds (purity > 95%) were purchased from ChemDiv.

Screening procedures

For assay of potentiator activity, FRT cells expressing ΔF508-CFTR and YFP were grown at 37 °C / 5% CO_2 for 18-24 h and then for 18-24 h at 27 °C, as described (Yang et al., 2003). At the time of the assay cells were washed with PBS and then incubated for 10 min with PBS (60 μL) containing forskolin (20 μM) and test compound (2.5 μM final concentration). Each well was assayed individually for iodide influx by recording fluorescence continuously (200 ms per point) for 2 s (baseline) and then for 12 s after rapid addition of 180 μL PBS in which 137 mM chloride was replaced by iodide. Fluorescence was recorded using a TECAN Infinite M1000 plate reader (TECAN Groups Ltd, Mannedorf, Switzerland) equipped with automated stacker and dual syringe pumps (excitation: 500 ± 10 nm; emission: 535 ± 10 nm). Initial iodide influx rate was computed as described (Ma et al, 2002). All compound plates contained negative controls (DMSO vehicle) and positive controls (50 μM genistein).

Chemiluminescence assays

CFBE cells expressing ΔF508-CFTR-HRP were incubated with 100 μL medium containing test compound, 3 μM VX-809 and 0.5 μg/ml doxycycline for 24 h at 37 °C. All plates contained negative controls (DMSO vehicle) and positive controls (3 μM VX-809). Cells were washed 4 times with PBS, and HRP activity was assayed by addition of 50 μL/well of HRP-substrate (WesternBright Sirius Kit,

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Advansta Corp, Menlo Park, CA), as described (Phuan et al., 2014). After shaking for 5 min, chemiluminescence was measured using a TECAN Infinite M1000 plate reader (integration time 100 ms).

Short-circuit current measurements

Short-circuit current measurements were done as previously described (Phuan et al., 2014). FRT cells expressing ΔF508-, wildtype- or G551D-CFTR were cultured on inserts (Snapwell; Corning, Lowell, MA) for 5-7 days and used when transepithelial resistance was > 2000 $\mu\Omega/cm^2$. For potentiator testing, the ΔF508-CFTR-expressing FRT cells were incubated for 18-24 h at 27 °C before measurements. The basolateral solution contained 120 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 25 mM $NaHCO_3$ and 5 mM HEPES, pH 7.4. In the apical bathing solution 60 mM NaCl was replaced by Na gluconate and $CaCl_2$ was increased to 2 mM. Solutions were bubbled with 95% O_2/5% CO_2 and maintained at 37 °C. The basolateral membrane was permeabilized with 250 μg/ml amphotericin B. Primary cultures of human bronchial epithelial cells were measured after 21-28 days of culture when cells reached full differentiation and transepithelial resistance was >1000 $\mu\Omega/cm^2$. The primary cultures were incubated with VX-809 (10 μM) at the basolateral side for 18-24 hours at 37 °C prior to measurements. The apical and basolateral chambers contained identical solutions: 130 mM NaCl, 0.38 mM KH_2PO_4, 2.1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose. Solutions were bubbled with 5% CO_2/95% O_2 and maintained at 37°C. Hemichambers were connected to a DVC-1000 voltage clamp (World Precision Instruments Inc., Sarasota, FL) via Ag/AgCl electrodes and 1 M KCl agar bridges for recording of short-circuit current.

CFTR plasma membrane density measurements

The plasma membrane density of 3HA-tagged CFTR was determined by cell surface ELISA, as described (Okiyoneda et al., 2010). Plasma membrane density measurements were normalized to cell viability determined by Alamar Blue assay (Invitrogen, Carlsbad, CA).

Immunoblotting

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Immunoblotting was performed as described (Okiyoneda et al., 2010). The 3HA-tagged ΔF508-CFTR was detected by monoclonal mouse anti-HA antibody (MMS101R, Covance), and monoclonal mouse anti-Na^+/K^+-ATPase antibody (H3, Santa Cruz Biotechnology) was used to confirm equal loading.

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RESULTS

Screening strategy

Fig. 1A,B diagrams the assays used to identify potentiators that do not interfere with ΔF508-CFTR corrector action. The primary high-throughput screen for potentiators was done using FRT cells stably expressing human ΔF508-CFTR and an iodide-sensitive YFP. The cells were cultured for 24 hours at 27 °C to target ΔF508-CFTR to the plasma membrane (low-temperature rescue). Iodide influx was measured by extracellular addition of iodide in the presence test compound (at 2.5 μM) and cAMP agonist (20 μM forskolin). Active potentiators identified in the primary screen were counter-screened for effect on corrector action using a human lung epithelium–derived (CFBE41o−) cell line (Ehrhardt et al., 2006) stably transfected with human ΔF508-CFTR containing a HRP inserted in its fourth extracellular loop (Phuan et al., 2014; Veit et al., 2014). The expression of wildtype-CFTR in CFBE is under the control of the same expression vector as in ΔF508-3HA and ΔF508-HRP cells reported previously (Veit et al., 2012), in which CFTR expression was shown to be similar to endogenous levels in Calu-3 cells. The ΔF508-HRP CFBE cells were cultured on 96-well plates and then potentiators (10 μM) were added for 18-24 hours together with VX-809 (3 μM). Cell surface ΔF508-CFTR was quantified by a luminescence readout. Potentiators that interfere with ΔF508-CFTR cellular stability or corrector action reduce luminescence signal. Fig. 1C shows reduced VX-809 corrector efficacy in cells following chronic exposure to VX-770 or genistein, but not P5, as reported before (Veit et al., 2014). Chemical structures are shown in Fig. 1D.

ΔF508-CFTR potentiators identified from screens

60,000 drug-like, synthetic small molecules were screened for potentiator activity, as well as 273 analogs of tetrahydrobenzothiophene P5. As summarized in Fig. 2A, 230 active compounds were identified from the 60,000-molecule screen and 38 active P5 analogs, with activity defined as > 50% of the maximal iodide influx produced by forskolin (20 μM) + genistein (50 μM). The fifty most active compounds were counter-screened for effect on corrector efficacy in VX-809-treated ΔF508-HRP CFBE cells, which produced 41 compounds from the 60,000-molecule screen and ten P5 analogs that did not

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significantly reduce luminescence signal. We choose the twelve most active compounds and grouped them into nine compound classes (Fig. 2B). Among the active compounds was a thiophene (compound G01) with structural similarity to tetrahydrobenzothiophene P5. In addition, structurally similar compounds included two 2-thioxo-4-amino-thiazoles (compounds A01 and A02) and three pyrazole-pyrrole- isoxazoles (compounds H01, H02 and H03). Supplemental Table 1 summarizes concentration-dependence data for selected compounds in ΔF508-CFTR FRT cells, with the most potent compounds showing EC_{50} ~ 0.03 μM for ΔF508-CFTR potentiation, without interference with VX-809 corrector action and with potentiator efficacy similar to maximal genistein.

Structure-activity analysis

Based on potency data, compounds of classes A, H and P5 were selected for further study. 189 commercially available class A analogs and 198 class H analogs were tested. Fig. 3A shows concentration-dependence data of selected compounds in ΔF508-CFTR FRT cells. Fig. 3B shows that these compounds do not significantly impair VX-809 corrector action in ΔF508-HRP CFBE cells. Supplemental Tables 2-4 summarize EC_{50} values for selective active compounds. Several class A analogs, including A03, A04 and A05, have similar or better EC_{50} than compounds A01 and A02 identified in the primary screen. The most active compound, A04, has an EC_{50} ~ 18 nM. For class H, only a few analogs showed EC_{50} similar the original compounds H01 and H02. Several P5 analogs were more potent than P5, including P11 and P12.

Structural determinants of activity for class A, H and P5 compounds are summarized in Fig. 3C. Correctors of class A, 2-thioxo-4-aminothiazoles, with the best activity have R^1 substituents of benzyl and 2-methyl-furan (Supplemental Table 1, A03 and A08). Reduced activity was seen with R^1 as halide-substituted benzene, alkyl-substituted-benzene, methyl, ethyl, phenethylene and allyl moieties. Interestingly, a wide array of substituents, including piperdine, pyrrolidine and substituted anilines, were tolerated at R^2, with the most active being R^2 as a simple ester (A04) and benzylamine (A03 and A05). Other rings, such as azepane, benzylpyridine and morpholine, reduced activity. Class H compounds contain three linked heterocycles – pyrazole-pyrrole-isoxazole. Analogs containing only two rings,

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such as pyrrole-isoxazole and pyrazole-isoxazole, were inactive, indicating that all three heterocycles are needed for activity. Limited substituents on the isoxazole were studied, with small substituents such as ethyl and methyl giving better activity, whereas bulky groups such as isopropyl reduced activity. The position at which the isoxazole linkage to pyrrole is crucial, as changing from the 3- to 2-position abolished activity. Changing the linker between the pyrazole and pyrrole rings from methylene (X: CH_2) to carbonyl (X: C=O) resulted in loss of activity. The substitution (R^2) on the pyrazole affected activity, with electron-poor rings such as 4-fluoro-benzene (H04) and 3-bromo-benzene (H05) given best activity, whereas electron-neutral (H06) or electronic-rich (H10) rings giving reduced activity.

The benzothiophene P5, which contains a 6-membered ring (n = 1), had greater potentiator activity than analogs with a 5-membered ring (n = 0) (P18) or a 7-membered ring (n = 2). At the R^1 position, the primary carboxamide ($CONH_2$) is crucial for activity, as replacing R^1 with cyano, ester, methyl-ketone, substituted-anilines, as well as substitutions on carboxamides, resulted in loss of activity. Similarly, substitution on the 2-position (R^2) of the tetrahydrobenzo ring with methyl and *t*-butyl also resulted in loss of activity. Alterations of R^3 gave more potent P5 analogs, including replacement of the 2-chlorobenzene in P5 by 2-fluorobenzene (P12) or 2-thiophene (P11). Close structural analogs of P5 with chloro-, methoxy- and nitro-groups substituted at different positions showed similar or better EC_{50} than P5. Changing R^3 from benzene to other substituted benzyl, biphenyl and phenoxy-phenyls reduced activity.

Biochemical studies of potentiator effect on ΔF508-CFTR expression

To biochemically confirm that chronic potentiator exposure does not interfere with ΔF508-CFTR plasma membrane expression, we used CFBE41o− cells stably expressing the 3HA-tagged ΔF508-CFTR (Sharma et al., 2004). 3HA-ΔF508-CFTR CFBE cells were treated with VX-770 and potentiators for 24 h. Fig. 4A (*left*) showed while VX-770 reduced ΔF508-CFTR plasma membrane expression, most of the potentiators did not affect ΔF508-CFTR expression, with the exception compounds H01 and H02. When the potentiators were incubated together with VX-809 (Fig. 4A, *center*) or VX-661 (Fig. 4A, *right*), most potentiators did not reduce ΔF508-CFTR PM expression.

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Compounds H01 and H02 reduced ΔF508-CFTR plasma membrane expression, although not as much as VX-770.

Potentiator effects on ΔF508-CFTR cellular expression were examined by immunoblot analysis. Potentiators were incubated alone, or with 3 μM VX-809 or 3 μM VX-661, in rΔF508-3HA-CFBE cells for 48 hours at 26 °C before lysis and immunoblot analysis (Fig. 4B). Densitometric analyses were done to quantify complex-glycosylated ΔF508-CFTR (C-band) in cell lysates (Fig. 4C). Most of the potentiator did not significantly decrease the amount of the complex-glycosylated ΔF508-CFTR (C-band), alone or in combination with VX-809 or VX-661. Several class H compounds (H01, H02 and H03) reduced the amount of the complex-glycosylated ΔF508-CFTR, similar to data in panel A. Two P5 analogs, P15 and P17, appeared to decrease complex-glycosylated ΔF508-CFTR in VX-661 but not VX-809 treated cells, suggesting that potentiator interference of corrector action might be corrector-dependent.

Short-circuit measurements in transfected FRT cells and in primary cultures of ΔF508/ΔF508 human bronchial epithelial cells

Based on the results of the biochemical and SAR studies, we choose the most potent class A and P5 analogs, A04 and P12, for further characterization by short-circuit current measurements. Apical membrane chloride current was measured in ΔF508-CFTR-expressing FRT cells, after low-temperature rescue, in response to A04 and P12, with data for VX-770 shown for comparison. Fig. 5A shows the concentration-dependent increases in apical membrane current seen in the presence of forskolin. Genistein produced a minimal increase in chloride current after maximal A04, P12 and VX-770. $CFTR_{inh}$-172 abolished all chloride current. As measured by short-circuit current the apparent EC_{50} for A04 and P12 potentiator activity were ~100 nM. The EC_{50} for VX-770 was ~30 nM, in agreement with published data (Van Goor et al., 2009). In side-by-side short-circuit current recordings (data not shown), maximal potentiator concentrations produced open probabilities of ~0.37 and ~0.34 for A04 and P12, respectively, as referenced against the open probability of ~0.40 produced by maximal VX-770 (Van Goor et al., 2009). A04 and P12 were also tested in FRT cells expressing wild-type CFTR (Fig.

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5B). A low concentration of forskolin (0.5 μM) was used to prevent fully activation of wild-type CFTR, which would mask potentiator action. After potentiator additions, 10 μM forskolin was added to fully activate wild-type CFTR, followed by 50 μM genistein, which had little effect, followed by 10 μM $CFTR_{inh}$-172, which inhibited all chloride current. A04 activated wild-type CFTR when added after 0.5 μM forskolin, while P12 showed partial activation, with apparent EC_{50} ~200 nM for both compounds. A04 and P12 were also tested in FRT cells expressing G551D-CFTR (Fig. 5C). G551D-CFTR produced little chloride current with maximal forskolin. A04 partially activated G551D-CFTR but only at high concentration (10 μM), while P12 showed minimal activation even at high concentration.

Short-circuit current measurements were also done in well-differentiated primary cultures of human bronchial epithelial cells from a ΔF508–homozygous subject. Short-circuit current was measured after chronic (18-24 hour) treatment with A04 or P12 together with VX-809. Cells incubated with 3 μM VX-809 alone showed increased short-circuit current in response to forskolin and VX-770, which was inhibited by $CFTR_{inh}$-172 (Fig. 6A *left*). Little current was seen in the absence of VX-809. Co-treatment with 5 μM A04 or P12 together with 3 μM VX-809 did not reduce chloride current when compared to VX-809 alone (Fig 6A *center*, *right*). Fig. 6B summarizes chloride current responses from data as in panel A, indicating that chronic treatment with A04 and P12 did not interfere with VX-809 corrector action. Finally, the potentiator action of A04 and P12 was tested in the primary cultures of ΔF508/ΔF508 human bronchial epithelial cells after VX-809 correction. Fig. 6C shows the A04 and P12 concentration-dependent increases in chloride current in the presence of forskolin. Genistein produced minimal increase in chloride current after maximal A04 or P12, and 10 μM $CFTR_{inh}$-172 abolished all chloride current. The apparent EC_{50} for A04 and P12 potentiator activity was ~300 nM in the ΔF508/ΔF508 human bronchial epithelial cells.

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DISCUSSION

This study was done to identify new potentiators that do not interfere with the action of ΔF508-CFTR corrrectors. This study was motivated by recent studies showing: (i) that the approved potentiator Ivacaftor (VX-770, Kalydeco) reduced correction efficacy of VX-809 and VX-661 (Veit et al., 2014; Cholon et al., 2014); (ii) that some but not all potentiators reduce correction efficacy (Veit et al., 2014); and (iii) combination VX-770 / VX-809 therapy showed limited clinical efficacy (Boyle et al., 2014; Vertex Pharmaceuticals press release, 2014). VX-770 has been approved to treat CF patients with defective CFTR gating but not cellular processing, including G551D-CFTR. However, the majority of CF patients carry the ΔF508 mutation in which the protein has defective gating as well as trafficking to the cell membrane. The limited clinical efficacy of combination VX-809/VX-770 therapy could perhaps be explained by in vitro data (Veit et al., 2014, Cholon et al., 2014) showing that VX-770 reduces the correction efficacy of VX-809 and VX-661 by increasing the turnover rate and destabilizing corrected ΔF508-CFTR. Thus, potentiators that do not interfere with corrector action or impair CFTR stability/turnover rate could have improved clinical benefit over VX-770 in combination therapy of CF caused by the ΔF508 mutation and potentially other mutations.

Our strategy to identify ΔF508-CFTR potentiators that do not interfere with corrector action utilized an initial, high-throughput functional, cell-based screen involving assay of iodide influx. Active compounds identified from the initial screen were counter-screened using a human lung epithelium-derived cell line expressing transfected HRP-tagged ΔF508-CFTR. The HRP-tag was engineered in the fourth extracellular loop for robust plate reader–based luminescence measurements of cell surface CFTR expression. A large number and variety of potentiators were identified, many of which did not interfere with corrector action. The most active potentiators, A04 and P12, were found to have nanomolar potency as shown by short-circuit current measurements in low-temperature rescued ΔF508-CFTR FRT cells and VX-809 treated ΔF508/ΔF08 human bronchial epithelial cells. Interestingly, P12 and A04 only minimally activated G551D-CFTR at high concentrations. Computational modeling has suggested that increased stability of G551D-CFTR compare to wildtype CFTR renders the G551D-CFTR protein too rigid and inflexible for channel gating (Serohijos et al.,

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2008). VX-770 mediated destabilization of G551D-CFTR might increase G551D-CFTR flexibility and result in restored channel gating function (Cholon et al., 2014). Our results with P12 and A04 suggest that these classes of compound potentiate CFTR gating most likely by a different mechanism of action from VX-770.

In addition to the functional and cell surface ELISA measurements of potentiator effect on corrector efficacy, which included studies on primary cultures of ΔF508/ΔF508 human bronchial cells, biochemical analyses were done to quantify total cellular ΔF508-CFTR and the accumulation of complex-glycosylated ΔF508-CFTR. Most compounds identified from the screen did not reduce the level of complex glycosylated ΔF508-CFTR, except several of class H compounds, which might preclude this class of compounds for further development. As noted, an interesting observation is that P16 and P17 appeared to decrease complex-glycosylated ΔF508-CFTR in VX-661 but not VX-809 or control cells. Potentiator interference of corrector action thus might be corrector-dependent. Specific potentiator-corrector combination might produce different therapeutic effect in different CFTR mutations. Thorough in vitro studies of potentiator-corrector combinations using pertinent CF mutations might be crucial before clinical trials. The modular multi-assay approach adopted in this study could facilitate the selection of potentiators for drug development.

Some 2-thioxo-4-aminothiazoles (class A) have reported antifungal (Chhabria et al., 2011) and antitubercular (Chhabria et al., 2014) activity. Reported biological activities for tetrahydrobenzothiophenes includes inhibition of tyrosine kinase receptor FLT3 (Patch et al., 2006) and influenza virus RNA polymerase assembly (Massari et al., 2013). Both thioxoaminothiazoles and tetrahydrobenzothiophenes can be prepared in 3-5 steps from commercially available starting materials, thus allowing facile and rapid diversification of these scaffolds for further medicinal chemistry and SAR efforts to improve biological activities and pharmacological properties. There are no biological reports on the exact pyrazole-pyrrole-isoxazole ring system as found in class H compounds. However, some pyrazoles with moderate structural similarity to class H have been reported to be 5-HT2A/5-HT2C receptor antagonist (Schadt et al., 2004), p38 kinase inhibitors (Naraian et al., 2005) and androgen receptor antagonists (Ito et al., 2009). To our knowledge, the channel-modulating effects of Class A

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and H compounds have not been reported.

In summary, the results here provide proof-of-concept for identification of nanomolar-potency potentiators that do not interfere with corrector action or CFTR stability.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Phuan, Veit, Lukacs, and Verkman.

Conducted experiments: Phuan, Veit, Tan.

Wrote or contributed to the writing of the manuscript: Phuan, Veit, Finkbeiner, Lukacs, and Verkman.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. **Screens for identification of ΔF508-CFTR potentiators that do not interfere with corrector action.** **(A)** Potentiator screen using FRT cells coexpressing ΔF508-CFTR and YFP iodide sensing protein. Cells were incubated at 27 ℃ for 24 h before assay to target ΔF508-CFTR to the plasma membrane. Test compounds at 2.5 μM were added for 10 min at room temperature in presence of forskolin (20 μM) before iodide addition. **(B)** Cell-surface expression screen using ΔF508-CFTR-HRP CFBE cells. Cells were incubated with test compounds and 3 μM VX-809 for 24 hours at 37 ℃. Cell-surface ΔF508-CFTR was assayed by a luminescence readout of HRP activity. **(C)** Luminescence readout of cell surface ΔF508-CFTR after 24-hour incubation with potentiators VX-770, P5 or genistein, together with 3 μM VX-809 (S.E., $n = 4$). **(D)** Chemical structures of potentiators.

Figure 2. **Screening results.** **(A)** Summary of screening results. **(B)** Chemical structures of nine classes of potentiators identified from the screen.

Figure 3. **Structure–activity relationships of class A, H and P5 potentiators.** **(A)** Concentration-dependence data of class A, H and P5 analogs in ΔF508-CFTR FRT cells (S.E., $n = 3$). **(B)** Cell-surface ΔF508-CFTR expression in CFBE cells following incubation with 10 μM class A or class H compounds or 2 μM P5 analogs, together with 3 μM VX-809 (S.E., $n = 4$). 100% luminescence signal is defined as background-subtracted luminescence signal of VX-809-corrected ΔF508-HRP CFBE cells. **(C)** Structural determinants of potentiator activity of class A, H and P5 compounds.

Figure 4. **Potentiator effects on ΔF508-CFTR plasma membrane expression.** **(A)** Plasma membrane expression of low-temperature rescued (26 °C, 48 h followed by 1 h chase at 37 °C) ΔF508-CFTR-3HA (rΔF508-3HA) in CFBE. Cells were treated with the indicated potentiators for 24 h (P11-17, A1-2, A4 - 1μM; H1-3, C1, D1, E1, F1– 5 μM) alone (*left*), or in the presence of 3 μM

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VX-809 (*center*) or 3 μM VX-661 (*right*). Data are expressed as percent of DMSO-treated controls (S.E., $n = 3$). **(B)** Representative immunoblots and **(C)** quantification of the potentiator effect on the expression pattern of rΔF508-3HA (26 °C, 48 h) in CFBE cells. Cells were treated with the indicated potentiators alone or in combination with 3 μM VX-809 or 3 μM VX-661 for 24 h at 26 ºC. CFTR was visualized with anti-HA antibody, anti-Na^+/K^+-ATPase antibody served as loading control. Densitometric analysis of complex-glycosylated (band C, empty arrowhead) ΔF508-CFTR is expressed as % of DMSO control (S.E., $n = 3$-9).

Figure 5. **Functional assays in FRT cells expressing ΔF508-, wildtype- and G551D-CFTR.** Representative short-circuit current measured in FRT cells expressing ΔF508-CFTR **(A)**, wildtype-CFTR **(B)** and G551D-CFTR **(C)** showing responses to indicated concentrations of forskolin (fsk), A04, P12 and VX-770. The ΔF508-CFTR expressing cells were incubated at 27 °C for 24 h before measurement. Genistein (gen, 50 μM) and $CFTR_{inh}$-172 (10 μM) were added where indicated.

Figure 6. **Functional assays in primary cultures of human bronchial epithelial cells from a homozygous ΔF508 CF patient.** **(A)** Cultures were incubated at 37 °C for 24 hours with 3 μM VX-809 (grey curve shows DMSO-vehicle treated cells) (*left*), 3 μM VX-809 + 5 μM A04 (*center*), or 3 μM VX-809 + 5 μM P12 (*right*). Amiloride (10 μM), VX-770 (10 μM), forskolin (20 μM) and $CFTR_{inh}$-172 (10 μM) were added where indicated. **(B)** Summary of changes in short-circuit current (ΔIsc) from experiments as in A (S.E.; $n = 3$ cultures each). **(C)** Short-circuit current measured in the ΔF508/ΔF508 human bronchial epithelial cells, showing responses to indicated A04 and P12 concentrations. Cells were incubated at 37 °C for 24 h with 3 μM VX-809 before measurement. Genistein (50 μM) and $CFTR_{inh}$-172 (10 μM) were added where indicated.

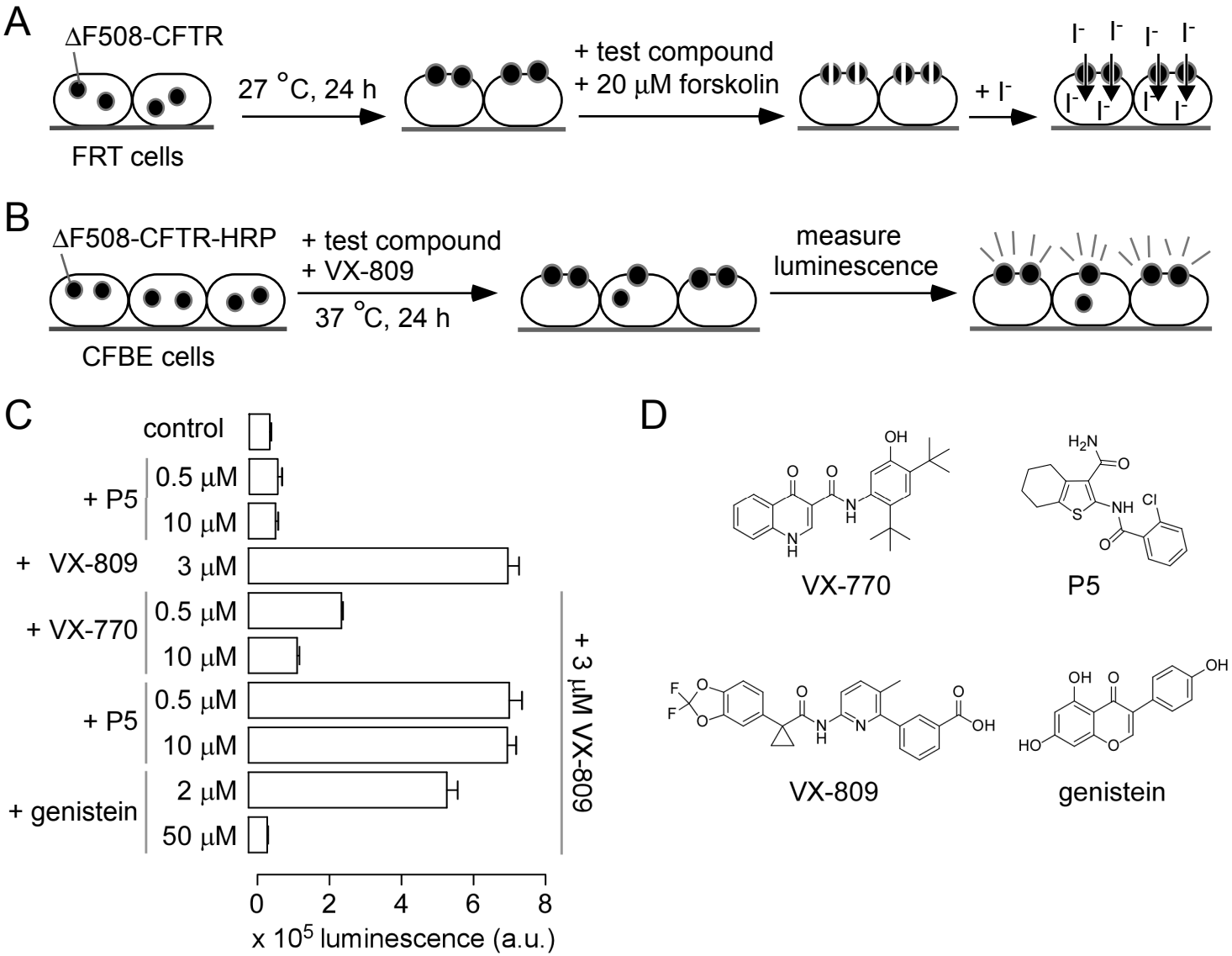

Figure 1

A

FRT ΔF508-CFTR
primary screen
60,000 compounds

↓

230 hits (>50 % maximal of genistein + forskolin)

↓

50 hits (tested with CFBE-ΔF508-CFTR-HRP assay)

↓

9 compound classes

FRT ΔF508-CFTR
463 P5 analogs

↓

38 hits (>50 % maximal of genistein + forskolin)

↓

10 hits (tested with CFBE-ΔF508-CFTR-HRP assay)

B

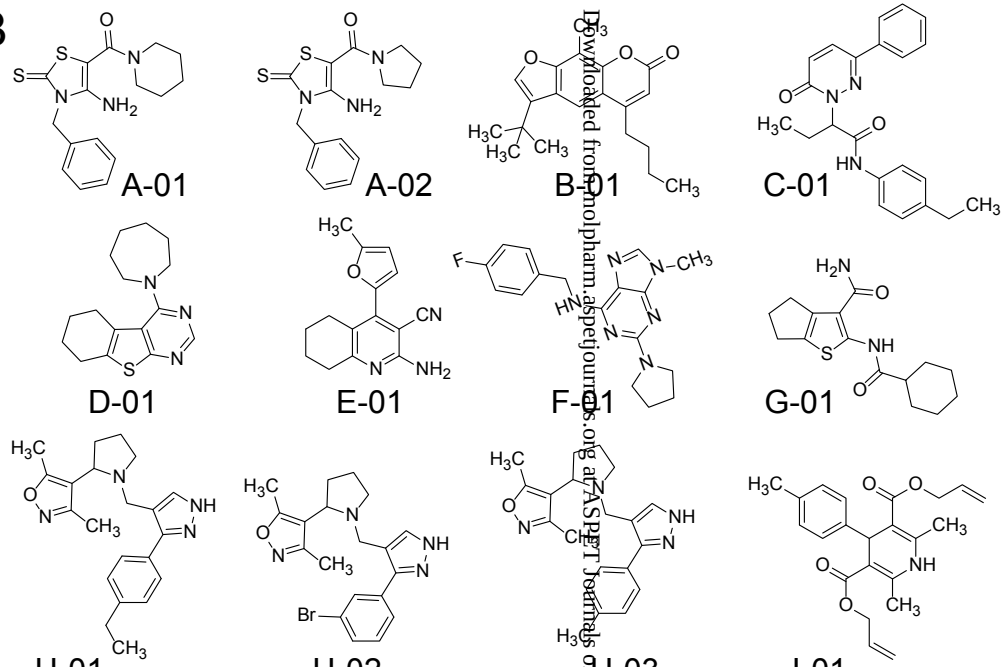

Figure 2

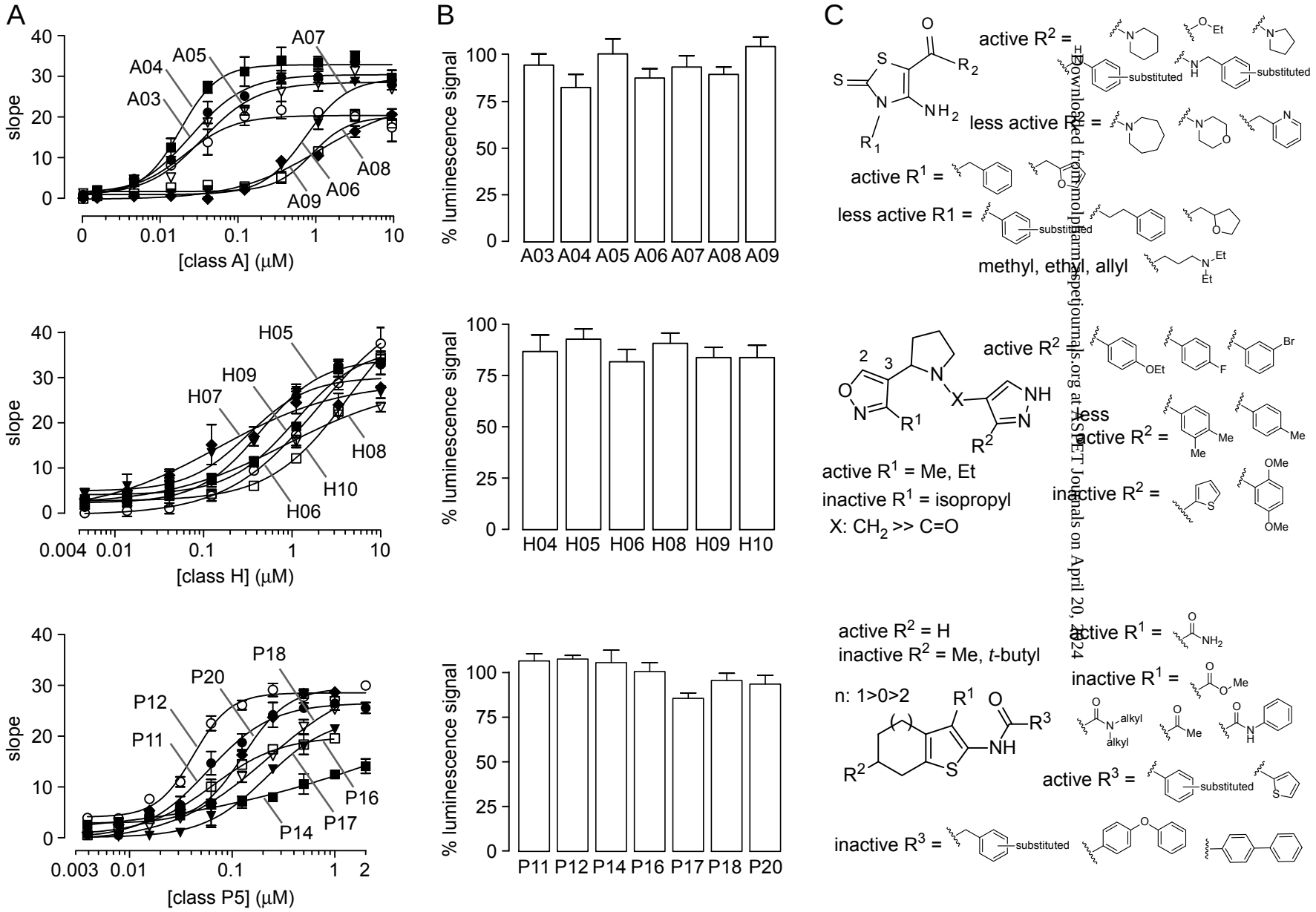

Figure 3

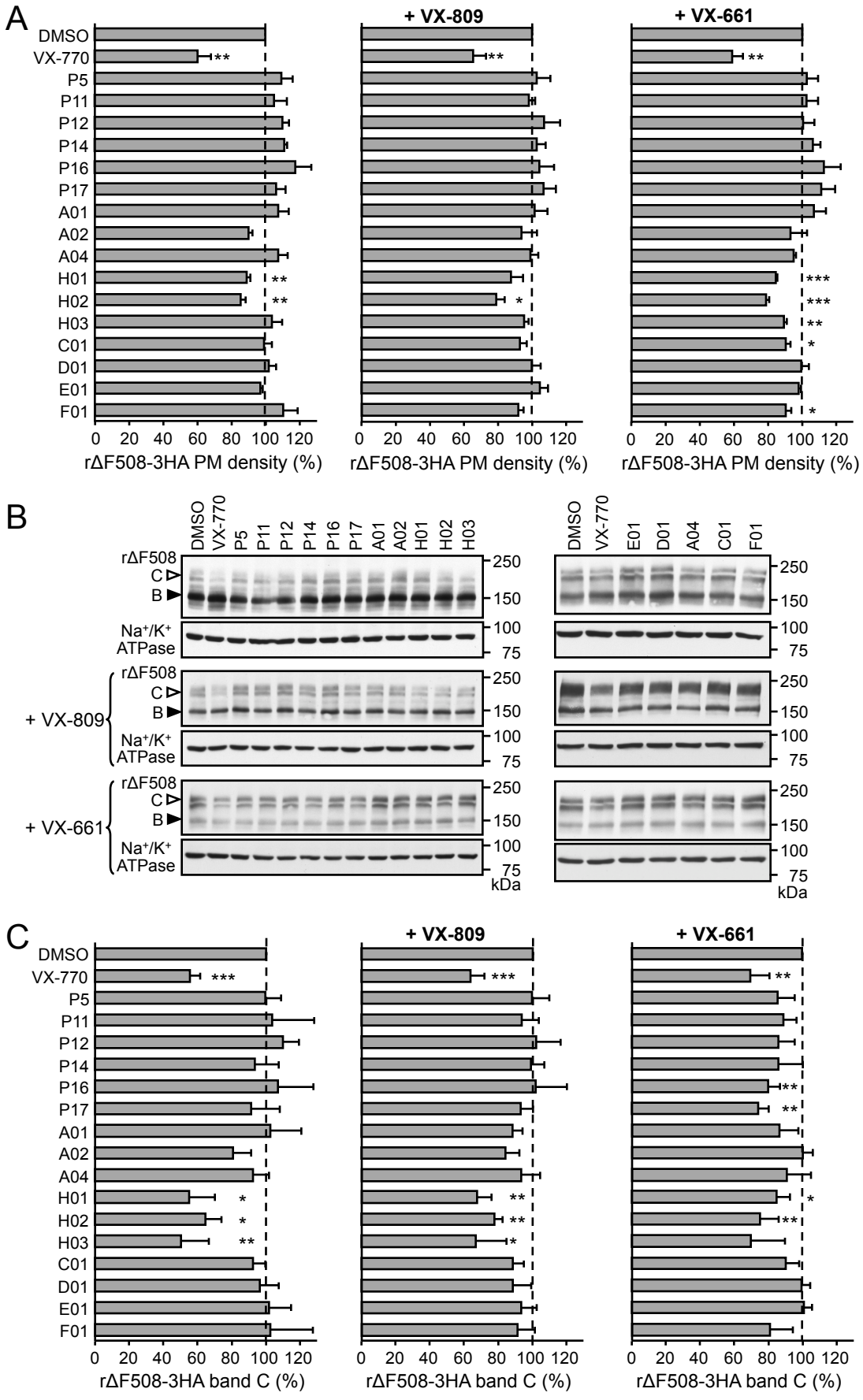
A
+ VX-809
+ VX-661
DMSO
VX-770
P5
P11
P12
P14
P16
P17
A01
A02
A04
H01
H02
H03
C01
D01
E01
F01
0 20 40 60 80 100 120
rΔF508-3HA PM density (%)
B
DMSO
VX-770
P5
P11
P12
P14
P16
P17
A01
A02
H01
H02
H03
E01
D01
A04
C01
F01
rΔF508
C
B
Na+/K+
ATPase
+ VX-809
+ VX-661
250
150
100
75
kDa
C
rΔF508-3HA band C (%)

Figure 4

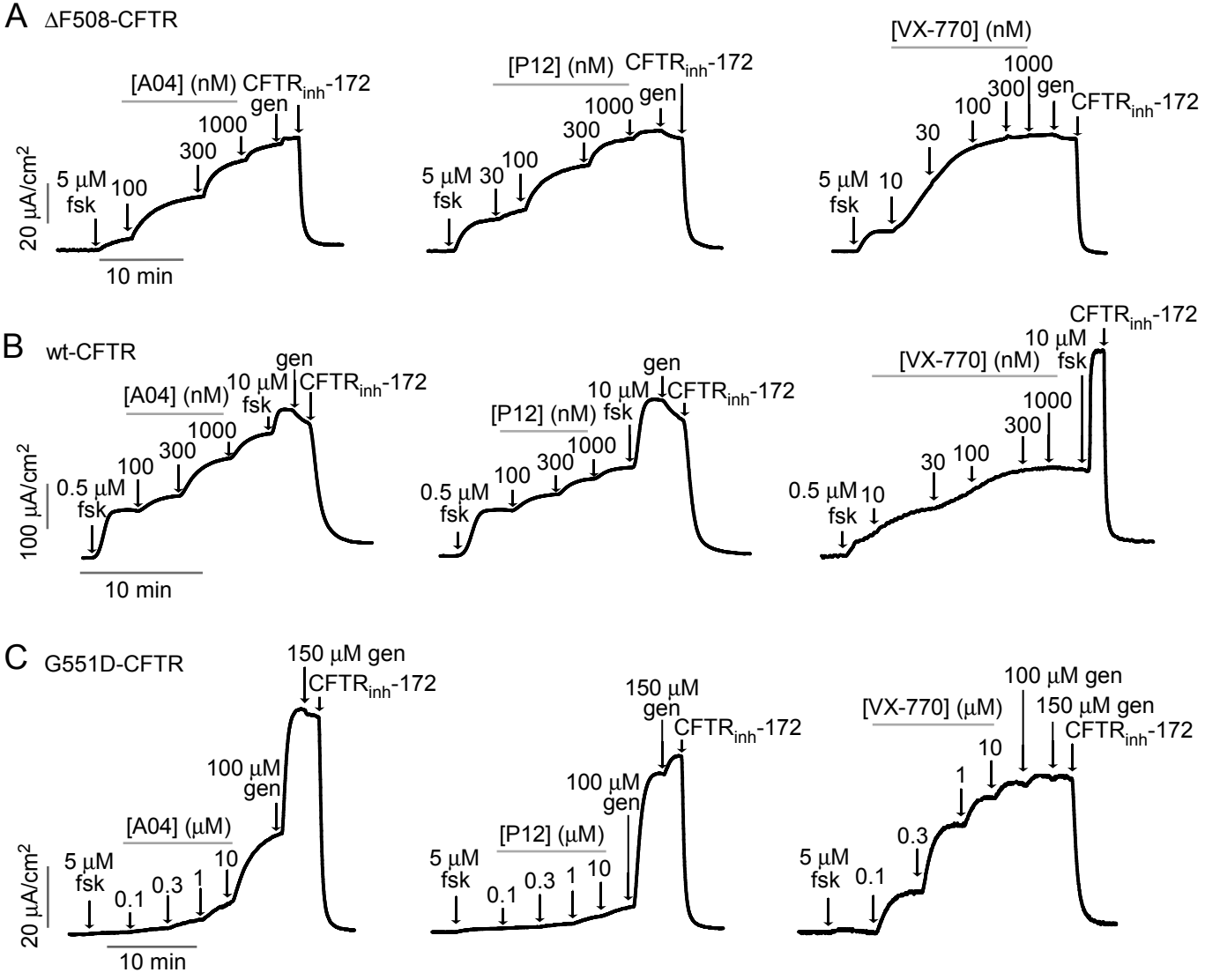
A ΔF508-CFTR
[A04] (nM)
CFTR$_{inh}$-172
gen
1000
300
100
5 μM
fsk
20 μA/cm^2
10 min
[P12] (nM)
[VX-770] (nM)
30
10
B wt-CFTR
10 μM
fsk
0.5 μM
100 μA/cm^2
C G551D-CFTR
150 μM gen
100 μM
gen
[A04] (μM)
[P12] (μM)
[VX-770] (μM)
0.1
0.3
1
10

Figure 5

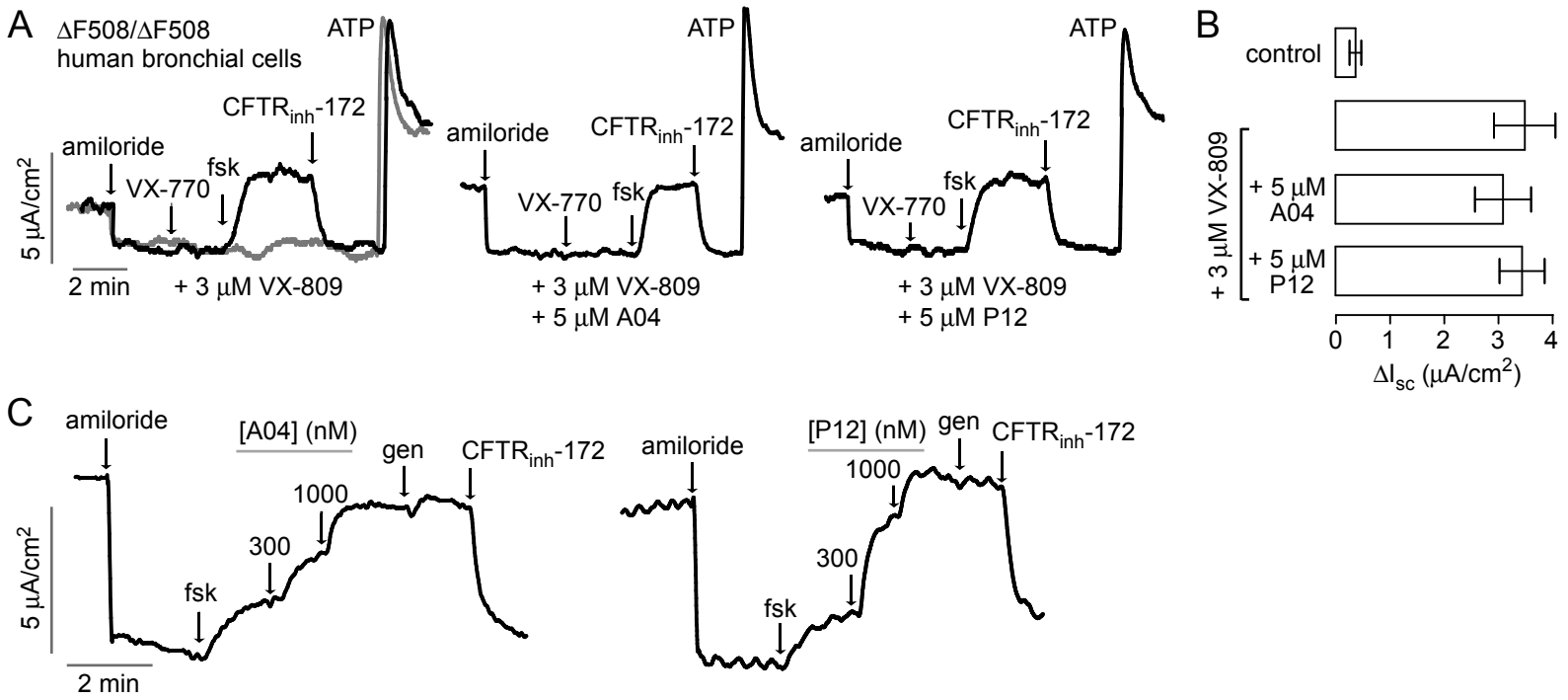
A
ΔF508/ΔF508
human bronchial cells
ATP
CFTRinh-172
amiloride
fsk
VX-770
5 μA/cm2
2 min
+ 3 μM VX-809
+ 3 μM VX-809
+ 5 μM A04
+ 3 μM VX-809
+ 5 μM P12
B
control
+ 3 μM VX-809
+ 5 μM A04
+ 5 μM P12
0 1 2 3 4
ΔIsc (μA/cm2)
C
amiloride
[A04] (nM)
gen
CFTRinh-172
1000
300
fsk
5 μA/cm2
2 min
[P12] (nM)

Figure 6