Novel Amino-Carbonitrile-Pyrazole Identified in a Small Molecule Screen Activates Wild-Type and Δ F508 Cystic Fibrosis Transmembrane Conductance Regulator in the Absence of a cAMP Agonist

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ABSTRACT

Cystic fibrosis (CF) is caused by loss-of-function mutations in the CF transmembrane conductance regulator (CFTR) Cl^- channel. We developed a phenotype-based high-throughput screen to identify small-molecule activators of human airway epithelial Ca^{2+} -activated Cl^- channels (CaCCs) for CF therapy. Unexpectedly, screening of $\sim 110,000$ synthetic small molecules revealed an amino-carbonitrile-pyrazole, C_{act} -A1, that activated CFTR but not CaCC Cl^- conductance. C_{act} -A1 produced large and sustained CFTR Cl^- currents in CFTR-expressing Fisher rat thyroid (FRT) cells and in primary cultures of human bronchial epithelial (HBE) cells, without increasing intracellular cAMP and in the absence of a cAMP agonist. C_{act} -A1 produced linear whole-cell currents. C_{act} -A1 also activated Δ F508-CFTR Cl^- currents in low temperature-rescued Δ F508-CFTR-expressing FRT cells and CF-HBE cells (from homozygous Δ F508 patients)

in the absence of a cAMP agonist, and showed additive effects with forskolin. In contrast, *N*-(2,4-di-*tert*-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (VX-770) and genistein produced little or no Δ F508-CFTR Cl^- current in the absence of a cAMP agonist. In FRT cells expressing G551D-CFTR and in CF nasal polyp epithelial cells (from a heterozygous G551D/Y1092X-CFTR patient), C_{act} -A1 produced little Cl^- current by itself but showed synergy with forskolin. The amino-carbonitrile-pyrazole C_{act} -A1 identified here is unique among prior CFTR-activating compounds, as it strongly activated wild-type and Δ F508-CFTR in the absence of a cAMP agonist. Increasing Δ F508-CFTR Cl^- conductance by an “activator,” as defined by activation in the absence of cAMP stimulation, provides a novel strategy for CF therapy that is different from that of a “potentiator,” which requires cAMP elevation.

Introduction

Cystic fibrosis (CF), the most common lethal genetic disease in the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) that impair its function as an epithelial cell Cl^- channel in the airways, pancreas, intestine, and other organs (Quinton, 1983; Wine, 1995; Riordan, 2008; Lukacs and Verkman, 2012). Several new approaches for drug therapy of CF have emerged, including Ivacaftor [VX-770; *N*-(2,4-di-*tert*-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide], a CFTR potentiator approved to treat CF patients with the G551D-CFTR mutation (Davis, 2011). Clinical trials of Ivacaftor in CF patients with the G551D mutation on at least

one CFTR allele showed improvements in lung function, pulmonary exacerbations, patient-reported respiratory symptoms, body weight, and sweat chloride concentration (Ramsey et al., 2011; Davis et al., 2012).

There is continued interest in the development of activators of wild-type (WT) CFTR, for potential therapy of constipation and some chronic pulmonary diseases, and of Δ F508-CFTR, the most common CF-causing CFTR mutation (Lacy and Levy, 2007; Verkman and Galiotta, 2009). Prior studies have revealed multiple chemical classes of CFTR activators that likely function by direct interaction with CFTR, including flavones/isoflavones, benzo[c]quinoliziniums, xanthines, benzimidazolones, fluorescein derivatives, and benzoflavones (Illek et al., 2000; Galiotta et al., 2001; Ma et al., 2002b; Caci et al., 2003; Springsteel et al., 2003). Various Δ F508-CFTR “potentiators” have been identified, including VX-770, phenylglycines, sulfonamides, and tetrahydrobenzothiophenes (Yang et al., 2003; Pedemonte et al., 2005; Van Goor et al., 2009).

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ABBREVIATIONS: BrdU, bromodeoxyuridine; CaCC, Ca^{2+} -activated Cl^- channel; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FRT, fisher rat thyroid; HBE, human bronchial epithelial; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; TMEM16A, transmembrane protein 16A; 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; WT, wild-type; YFP, yellow fluorescent protein.

Potentiators require a cAMP agonist to increase $\Delta F508$ -CFTR Cl^- current.

The original goal of this study was to perform a high-throughput screen to identify small-molecule activators of Ca^{2+} -activated Cl^- channels (CaCCs) in human airway epithelium. We previously reported small-molecule activators of a CaCC, transmembrane protein 16A (TMEM16A) (Namkung et al., 2011b); however, TMEM16A is expressed at low levels in unstimulated airway epithelium (Namkung et al., 2011a) and hence is not a good target for CF therapy. We therefore developed a phenotype screen to identify activators of non-TMEM16A CaCC(s) in the human epithelial cell line Calu-3. Calu-3 cells transfected with the yellow fluorescent protein-based halide indicator were incubated with test compounds and subjected to an inwardly directed I^- gradient. In an attempt to select for activators of non-CFTR Cl^- channels, the screen was done in the presence of the thiazolidinone CFTR inhibitor CFTR_{inh}-172. Unexpectedly, one of the strongest activators of I^- influx, the amino-carbonitrile-pyrazole C_{act}-A1, was found to be a CFTR activator that competed with CFTR_{inh}-172 and activated CFTR in a cAMP-independent manner, and, unlike prior CFTR activators, without the requirement of basal CFTR activation in primary cultures of human airway epithelial cells. Also, unlike prior potentiators including VX-770, C_{act}-A1 also activated $\Delta F508$ -CFTR in human CF airway epithelial cells without a cAMP agonist. The unique CFTR activation mechanism of C_{act}-A1 suggests a novel therapeutic alternative in CF caused by the $\Delta F508$ mutation—an “activator” rather than “potentiator.”

Materials and Methods

Forskolin, genistein, and other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). VX-770 was purchased from Selleck Chemicals (Houston, TX). CFTR_{inh}-172 was synthesized as described elsewhere (Ma et al., 2002a). C_{act}-A1 was purchased from ChemDiv (San Diego, CA). The compound collections

used for screening included ~100,000 synthetic small molecules from ChemDiv and Asinex (San Diego, CA), and ~7500 purified natural products from Analyticon (Potsdam, Germany), Timtek (Newark, NJ), and Biomol (Plymouth Meeting, PA). Compounds were maintained as dimethylsulfoxide stock solutions. The HCO_3^- -buffered solution contained (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4). In the half- Cl^- solution 65 mM NaCl in the HCO_3^- -buffered solution was replaced by Na gluconate.

Cell Culture. Calu-3 cells were maintained in Dulbecco's modified Eagle's medium/F-12 (1:1) medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu g/ml$ streptomycin. Calu-3 cells were stably transfected with the halide sensor yellow fluorescent protein (YFP)-H148Q/I152L/F46L. Fisher rat thyroid (FRT) cells expressing human WT-, $\Delta F508$ -, and G551D-CFTR, and TMEM16A (abc) were grown in F-12 Modified Coon's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu g/ml$ streptomycin. Primary cultures of non-CF and CF human airway epithelial cells were grown at an air-liquid interface as described (Levin et al., 2006). Cells were plated at a density of 5×10^5 per cm^2 onto 12-mm diameter, 0.4- μm pore polycarbonate cell culture inserts (Snapwell; Corning, Lowell, MA) precoated with human placental collagen (15 $\mu g/cm^2$; Sigma-Aldrich). Cultures were grown at an air-liquid interface in air-liquid interface (ALI) medium at 37°C in 5% $CO_2/95\%$ air (Fulcher et al., 2005). The medium was changed every 2–3 days. Cultures were used 21–30 days after plating, at which time transepithelial resistance was 400–1000 Ωcm^2 and an ASL film was seen.

Cell-Based High-Throughput Screening. Calu-3-YFP cells were plated in 96-well black-walled microplates (Corning Inc., Corning, NY) at a density of 20,000 cells per well in F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 $\mu g/ml$ streptomycin. To increase CaCC current, interleukin-4 (10 ng/ml) was added at 24 hours after plating, and the cells were incubated for an additional 48 hours. Assays were done using an automated screening platform equipped with Infinite F500 and Infinite M1000 PRO fluorescence plate readers (Tecan, Durham, NC). Each well of a 96-well plate was washed 3 times in phosphate-buffered saline (PBS) (200 $\mu l/wash$), leaving 50 μl PBS including 10 μM CFTR_{inh}-172. Test compounds (0.5 μl) were added to each well

Fig. 1. Phenotype screen for identification of small-molecule Cl^- channel activators in Calu-3 cells. (A) Fluorescence micrograph image of Calu-3 cells stably expressing the halide-sensitive cytoplasmic fluorescent sensor YFP-H148Q/I152L/F46L. (B) Time course of YFP fluorescence after extracellular I^- addition. As indicated, solutions contained vehicle, CFTR_{inh}-172 (10 μM) with forskolin (10 μM) or ionomycin (1 μM), and forskolin (10 μM), (mean $\pm$ S.E., $n = 4$). (C) Screening protocol. Calu-3 cells stably expressing the halide-sensitive YFP were preincubated with CFTR_{inh}-172 and then incubated for 10 minutes with test compound. Fluorescence was monitored in response to addition of iodide. (D) Fluorescence measured in single wells of 96-well plates, showing examples of inactive and active compounds.

at a 25 μM final concentration. After 5 minutes, 96-well plates were transferred to a plate reader preheated to 37°C for fluorescence assay. Each well was assayed individually for CaCCs-mediated I^- influx by recording fluorescence continuously (200 millisecond per point) for 2 seconds (baseline), then 50 μl of 140 mM I^- solution was added at 2 seconds and then YFP fluorescence was recorded for 6 seconds. The initial rate of I^- influx following each of the solution additions was computed from fluorescence data by nonlinear regression.

Short-Circuit Current. Snapwell inserts containing CFTR-expressing FRT or primary culture of human airway cells were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA). Forskolin, genistein, VX-770, CFTR_{inh}-172, and C_{act}-A1 were added to the apical and basolateral bath solution. For primary cultures of human airway cells, symmetrical HCO_3^- -buffered solutions were used, and epithelial sodium channel (ENaC) was inhibited by pretreatment with amiloride (100 μM). For FRT cells, the apical bath was filled with a half- Cl^- solution, and the basolateral bath was filled with HCO_3^- -buffered solution, and the basolateral membrane was permeabilized with 250 $\mu\text{g}/\text{ml}$ amphotericin B. All cells were bathed for a 10-minute stabilization period and aerated with 95% $\text{O}_2/5\%$ CO_2 at 37°C, except for FRT cells expressing WT-CFTR, which were bathed at room temperature. Apical membrane current (for FRT cells) and short-circuit current were measured using an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, FL) and recorded using PowerLab/8sp (AD Instruments, Castle Hill, NSW, Australia).

Patch-Clamp. Whole-cell patch-clamp recordings were performed on CFTR-expressing FRT cells. The bath solution contained (in mM): 140 NMDG-Cl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM): 130 CsCl, 0.5 EGTA, 1 MgCl_2 ,

1 Tris-ATP, and 10 HEPES (pH 7.2). Pipettes were pulled from borosilicate glass and had resistances of 3–5 M Ω after fire polishing. Seal resistances were between 3 and 10 G Ω . After establishing the whole-cell configuration, CFTR was activated by forskolin and/or C_{act}-A1. Whole-cell currents were elicited by applying hyperpolarizing and depolarizing voltage pulses from a holding potential of 0 mV to potentials between –80 and +80 mV in steps of 20 mV. Recordings were made at room temperature using an Axopatch-200B (Axon Instruments). Currents were digitized with a Digidata 1440A converter (Axon Instruments), filtered at 5 kHz, and sampled at 1 kHz.

Intracellular Calcium Measurement. FRT cells expressing human TMEM16A were cultured in 96-well black-walled microplates and loaded with Fluo-4 NW per the manufacturer's protocol (Invitrogen, Carlsbad, CA). Fluo-4 fluorescence was measured with a FLUOstar Optima fluorescence plate reader equipped with syringe pumps and custom Fluo-4 excitation/emission filters (485/538 nm).

cAMP Assay. Human bronchial epithelial (HBE) cells grown on permeable supports and FRT cells grown on 12-well culture plates were washed 3 times with PBS at 37°C and then incubated in PBS at 37°C containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) for 5 minutes in the absence or presence of forskolin/C_{act}-A1. After 10-minutes incubation, the cells were washed with cold PBS and cytosolic cAMP was measured using a cAMP immunoassay kit (Parameter cAMP Immunoassay Kit; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Cell Proliferation Assays. Calu-3 cells (human lung epithelial cells) were seeded (5000 cells/well) on 96-well black-walled microplates. After 24 hours of incubation, cells were treated with different concentrations of C_{act}-A1 (0, 3, 10, 30 μM) and then incubated for 2 days. The culture medium was changed every 12 hours, and the cells

Fig. 2. Chemical structures of CFTR activators. (A) Structures shown of the most potent CFTR activator of each of four classes (C_{act}-X1, where X = A, B, C, or D). (B) Short-circuit (apical membrane) current measured in WT-CFTR expressing FRT cells in the presence of a trans-epithelial chloride gradient and after basolateral membrane permeabilization. Representative current traces showing forskolin (20 μM), C_{act}-A1, C_{act}-B1, C_{act}-C1, or C_{act}-D1 stimulated CFTR Cl^- current. Cl^- current was inhibited by addition of 20 μM CFTR_{inh}-172.

were treated with C_{act} -A1. To assess cell proliferation, methanethiosulfonate and bromodeoxyuridine (BrdU) assays were done using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Fitchburg, WI) and Cell Proliferation Enzyme-Linked Immunosorbent Assay, BrdU (colorimetric) kit (Roche Applied Science, Indianapolis, IN), respectively.

Results

Identification of CFTR Activators. A cell-based phenotype screen was developed to identify activators of Ca^{2+} -activated Cl^{-} channels in human airway epithelia. Cl^{-} channel activity was measured in Calu-3 cells, a human airway epithelial cell line that natively expresses CFTR and CaCC(s) (Wine et al., 1994; Namkung et al., 2011a). Calu-3 cells were stably transfected with the genetically encoded I^{-} -sensing fluorescent protein, YFP-F46L/H148Q/I152L, yielding highly fluorescent cells (Fig. 1A). Using a fluorescence plate reader assay in which I^{-} was added to cells after preincubation with agonists/inhibitors, the CFTR inhibitor CFTR_{inh}-172 blocked forskolin-stimulated, CFTR-dependent I^{-} influx, but not ionomycin-induced I^{-} influx, which involves CaCC activation (Fig. 1B). For

screening to identify CaCC activators, the Calu-3 cells were preincubated with CFTR_{inh}-172 and test compounds in PBS before addition of an I^{-} -containing solution (Fig. 1C). I^{-} addition produced little YFP fluorescence quenching in the absence of the activators because of the low basal I^{-} permeability of Calu-3 cells. Examples of original screening data are shown in Fig. 1D.

Screening of ~110,000 small synthetic molecules and natural products yielded 46 compounds that at 10 μ M increased I^{-} influx by 70% or more compared with that produced by ionomycin. Secondary testing of the 46 compounds by short-circuit current measurement in interleukin-4-treated Calu-3 cells showed that nine of compounds increased the Cl^{-} current, of which five weakly activated the native CaCC Cl^{-} current and, unexpectedly, four strongly activated CFTR Cl^{-} current, even in the absence of a cAMP agonist (Fig. 2). The four CFTR activators, which were identified in the primary screen done in the presence of 5 μ M CFTR_{inh}-172, probably compete with CFTR_{inh}-172 for binding to CFTR. Of the four CFTR activators, C_{act} -A1 was further studied because it most strongly activated CFTR-dependent Cl^{-} current in primary cultures of human bronchial epithelial cells.

Fig. 3. Characterization of C_{act} -A1, a small-molecule CFTR activator. (A) C_{act} -A1 concentration-dependent activation of CFTR in FRT cells expressing human WT-CFTR (mean $\pm$ S.E., $n = 4-7$). (B) C_{act} -A1 reversibility. C_{act} -A1 (10 μ M) addition followed by washout and readdition. The dashed line shows C_{act} -A1-induced Cl^{-} current without washing. (C) CFTR_{inh}-172 dose-response for inhibition of CFTR chloride current in FRT cells expressing WT-CFTR. CFTR was stimulated by 20 μ M forskolin (closed circles) or 30 μ M C_{act} -A1 (open circles). (D) Short-circuit current (left) and intracellular calcium concentration (right) measured in FRT cells expressing TMEM16A. TMEM16A was activated by 100 μ M ATP. (E) Short-circuit current measured in HBE cells. (Left) CFTR was activated by 20 μ M forskolin and inhibited by 20 μ M CFTR_{inh}-172. (Right) CFTR was activated by indicated concentrations of C_{act} -A1. The epithelial sodium channel (ENaC) was inhibited by pretreatment with 10 μ M amiloride. (F) cAMP accumulation in FRT and HBE cells in response to addition of C_{act} -A1 (10 μ M) and forskolin (10 μ M) (mean $\pm$ S.E., $n = 3-4$). (G) Calu-3 cells were treated with the indicated concentrations of C_{act} -A1, and cell proliferation was measured after 2 days using methanethiosulfonate (MTS) and BrdU assays (mean $\pm$ S.E., $n = 6$).

C_{act}-A1 Reversibly Activates WT-CFTR in Human Bronchial Epithelial Cells. Short-circuit current measurements in CFTR-expressing FRT cells gave an EC₅₀ of ~1.6 μ M for C_{act}-A1 (Fig. 3A). Figure 3B shows that C_{act}-A1 activation is reversible, as anticipated. Figure 3C shows a CFTR_{inh}-172 concentration-dependence for inhibition of CFTR Cl⁻ current in FRT cells expressing WT-CFTR, comparing the results for stimulation by forskolin versus C_{act}-A1. The IC₅₀ for CFTR_{inh}-172 inhibition was 2.6-fold greater when stimulated by C_{act}-A1 (IC₅₀ 0.37 μ M) than with forskolin (IC₅₀ 0.14 μ M). To investigate whether C_{act}-A1 affects TMEM16A, a CaCC, or intracellular calcium concentration, short-circuit current, and intracellular calcium concentration were measured in FRT cells expressing human TMEM16A. C_{act}-A1 did not alter TMEM16A function or intracellular calcium concentration (Fig. 3D). Short-circuit current measurements in HBE cells showed strong CFTR activation by C_{act}-A1, which was inhibited by CFTR_{inh}-172 (Fig. 3E). The activation of CFTR produced by maximal C_{act}-A1 in the absence of forskolin was comparable to that produced by maximal forskolin. Figure 3F shows that C_{act}-A1 did not increase intracellular cAMP concentration in FRT cells or in primary cultures of HBE cells. C_{act}-A1 at up to 30 μ M showed no cytotoxicity as measured by methanethiosulfonate and BrdU assays (Fig. 3G). C_{act}-A1 thus reversibly activates CFTR in human airway

epithelial cells without elevation of intracellular cAMP and without the need for a cAMP agonist.

To further characterize CFTR activation by C_{act}-A1, the effect of low concentrations of forskolin on C_{act}-A1-induced CFTR activation was studied, recognizing that most CFTR activators, including flavones and benzimidazolones, require a basal level of CFTR phosphorylation for activation (Galletta et al., 2001). Figure 4, A–C, shows additive effects of submaximal C_{act}-A1 and forskolin. In FRT cells expressing WT-CFTR, a high concentration of VX-770 (30 μ M) produced little CFTR Cl⁻ current, whereas C_{act}-A1 strongly activated CFTR Cl⁻ current (Fig. 4D). Whole-cell patch-clamp measurements showed that CFTR activation by maximal C_{act}-A1 produced a linear current/voltage relationship, similar in magnitude to that produced by maximal forskolin activation (Fig. 4E).

C_{act}-A1 Activates and Potentiates Δ F508-CFTR. A unique property of C_{act}-A1 was its ability to activate Δ F508-CFTR Cl⁻ current in the absence of forskolin, which was demonstrated in Δ F508-CFTR expressing FRT and CF-HBE cells. In these studies, Δ F508-CFTR was rescued by 24-hours' incubation at low temperature to promote plasma membrane targeting. In FRT cells, maximal C_{act}-A1 produced Cl⁻ current comparable to that of maximal forskolin, which was ~50% of that produced by maximal C_{act}-A1 and forskolin

Fig. 4. Additive effects of submaximal C_{act}-A1 and forskolin for activation of WT-CFTR. (A and B) Short-circuit current measured in FRT cells expressing WT-CFTR. CFTR was activated by indicated concentrations of C_{act}-A1 and forskolin. CFTR current was inhibited by 20 μ M CFTR_{inh}-172. (C) CFTR activation with indicated combinations of C_{act}-A1 and forskolin (mean $\pm$ S.E., $n = 3-5$), with 100% inactivation defined as that produced by 20 μ M forskolin alone. (D) Short-circuit current measured in FRT cells expressing WT-CFTR. CFTR was stimulated by VX-770, and C_{act}-A1 and 20 μ M forskolin. (E) Whole-cell CFTR Cl⁻ currents were recorded at a holding potential at 0 mV, and pulsing to voltages between ± 80 mV (in steps of 20 mV) in FRT cells expressing WT-CFTR. (Right) Summary of current density data measured at +80 mV (mean $\pm$ S.E., $n = 4-6$).

together (Fig. 5A). Interestingly, similar current was seen with C_{act} -A1 and VX-770 in the absence of forskolin. Figure 5B shows measurements done with different combinations of C_{act} -A1, forskolin, VX-770, and genistein. Maximal VX-770 (gray line) and genistein (dashed line), each alone, produced little $\Delta F508$ -CFTR activation in the absence of forskolin pretreatment, whereas C_{act} -A1 alone (black line) produced strong activation. Combined application of C_{act} -A1, genistein, or VX-770 with forskolin significantly activated $\Delta F508$ -CFTR compared with forskolin alone. Therefore, in contrast to the potentiator VX-770, C_{act} -A1 functions as both a $\Delta F508$ -CFTR activator (effective without a cAMP agonist) and potentiator (effective with a cAMP agonist). Similar measurements were done in HBE cells cultured from airways of $\Delta F508$ homozygous patients. As found in FRT cells expressing $\Delta F508$ -CFTR, C_{act} -A1 not only activated $\Delta F508$ -CFTR in the absence of a cAMP agonist, but also potentiated forskolin-induced Cl^- current by more than 2-fold. The EC_{50} of C_{act} -A1 for activation of $\Delta F508$ -CFTR was $3.5 \mu M$ in the primary CF-HBE cell

cultures (Fig. 5C). Whole-cell patch-clamp in $\Delta F508$ -CFTR expressing FRT cells showed that C_{act} -A1 strongly potentiated forskolin-induced Cl^- current without altering the linear current/voltage relationship of activated $\Delta F508$ -CFTR (Fig. 5D).

C_{act} -A1 Also Activates and Potentiates G551D-CFTR. C_{act} -A1 activation of the CF-causing gating mutant G551D-CFTR was investigated in G551D-CFTR expressing FRT cells. C_{act} -A1 ($30 \mu M$) and forskolin ($20 \mu M$), each alone, produced weak activation of G551D-CFTR (Fig. 6, A and B). However, combined application of C_{act} -A1 and forskolin showed synergistic activation of G551D-CFTR, albeit much smaller than the potentiation effect of VX-770. VX-770 ($10 \mu M$) strongly increased both forskolin- and C_{act} -A1-stimulated G551D-CFTR Cl^- current. Interestingly, C_{act} -A1 potentiated the G551D-CFTR Cl^- current activated by maximal forskolin with VX-770 (Fig. 6A, top left). Figure 6C shows whole-cell patch-clamp recordings in G551D-CFTR expressing FRT cells. VX-770 strongly potentiated C_{act} -A1- or forskolin-induced

Fig. 5. C_{act} -A1 activates and potentiates low-temperature rescued $\Delta F508$ -CFTR. (A) Short-circuit current measured in FRT cells expressing human $\Delta F508$ -CFTR. $\Delta F508$ -CFTR was rescued by low temperature (27°C) incubation for 24 hours. Where indicated, additions were done of $30 \mu M$ C_{act} -A1, $20 \mu M$ forskolin and $10 \mu M$ VX-770. (B) (Left) C_{act} -A1 ($30 \mu M$), genistein ($50 \mu M$) or VX-770 ($10 \mu M$) were added, followed by forskolin ($20 \mu M$). (Right) Summary of short-circuit current increases (ΔI_{sc} , mean $\pm$ S.E., $n = 4-6$). * $P < 0.05$; ** $P < 0.001$. (C) (Left) Short-circuit current in CF-HBE cells from $\Delta F508$ homozygous CF patients. Following low-temperature rescue, $\Delta F508$ -CFTR was stimulated by C_{act} -A1 and forskolin and then inhibited by $20 \mu M$ $CFTR_{inh}$ -172. The bar graph summarizes short-circuit current data (mean $\pm$ S.E., $n = 3-5$). (Right) C_{act} -A1 concentration-dependent activation of $\Delta F508$ -CFTR in CF-HBE cells (mean $\pm$ S.E., $n = 3$). (D) Whole-cell $\Delta F508$ -CFTR currents were recorded at a holding potential at 0 mV, and pulsing to voltages between ± 80 mV (in steps of 20 mV) in $\Delta F508$ -CFTR expressing FRT cells. (Right) Summary of current density data measured at +80 mV (mean $\pm$ S.E., $n = 4-6$).

Fig. 6. G551D-CFTR is weakly activated but strongly potentiated by C_{act}-A1 in G551D-CFTR expressing FRT cells. (A) G551D-CFTR was stimulated by application of 30 μM C_{act}-A1, 20 μM forskolin, and 10 μM VX-770. G551D-CFTR-dependent current was inhibited by 20 μM CFTR_{inh}-172. (B) Summary of peak currents (mean ± S.E., *n* = 4–7). (C) Whole-cell G551D-CFTR currents were recorded at a holding potential at 0 mV, and pulsing to voltages between ±80 mV (in steps of 20 mV) in G551D-CFTR-expressing FRT cells. Bottom right: summary of current density data measured at +80 mV (mean ± S.E., *n* = 4–7). **P* < 0.05; ***P* < 0.001.

Cl⁻ current. C_{act}-A1 significantly increased G551D-CFTR current produced by maximal forskolin and VX-770, in agreement with the short-circuit current data.

We also measured short-circuit current in primary cultures of human nasal polyp epithelial cells generated from a compound heterozygous CF patient with G551D/Y1092X-CFTR mutations. The Y1092X mutation is a nonsense mutation that does not produce functional protein (Bozon et al., 1994). Figure 7 shows significant synergy between C_{act}-A1 and forskolin, with the maximal current produced by VX-770 and forskolin further increased by C_{act}-A1.

Discussion

Screening of ~110,000 synthetic small molecules and natural products identified the novel amino-carbonitrile-pyrazole CFTR activator C_{act}-A1, which activated WT- and ΔF508-CFTR Cl⁻ currents without cAMP elevation and without the need for a cAMP agonist. Though C_{act}-A1 produced linear Cl⁻ CFTR currents, its activity in the absence of a cAMP agonist, in contrast to existing activators and potentiators such as genistein and VX-770, suggests a novel mechanism of action. C_{act}-A1 or other CFTR “activators” are potential

alternatives for therapy of CF caused by the ΔF508 mutation and, potentially, some gating mutations.

Recent analysis of VX-770 activation of CFTR suggests two distinct mechanisms—an ATP-dependent and an unconventional (ATP-independent) mechanism. VX-770 not only increases the open time of WT-CFTR by stabilizing a posthydrolytic open state in an ATP-dependent manner, but also increases the spontaneous ATP-independent opening rate of CFTR (Eckford et al., 2012; Jih and Hwang, 2013). The binding site of VX-770 is not known, though there are clues that it may bind to the transmembrane domains of CFTR (Jih and Hwang, 2013). The different properties of C_{act}-A1 versus VX-770 with regard to CFTR activation in the absence of a cAMP agonist suggest different mechanisms of action. cAMP agonists activate CFTR by phosphorylation of its regulatory domain; however, C_{act}-A1 does not elevate intracellular cAMP. As shown in Fig. 3C, C_{act}-A1 competes with CFTR_{inh}-172, a CFTR-selective inhibitor, and a high concentration of VX-770 (30 μM) produced little CFTR Cl⁻ current, whereas C_{act}-A1 strongly activated CFTR Cl⁻ current without forskolin (Fig. 4D). Further, the additive effects of C_{act}-A1 and forskolin for ΔF508-CFTR (Fig. 5A) suggest direct interaction between CFTR and C_{act}-A1, as well as an unconventional mechanism of CFTR activation.

Fig. 7. C_{act} -A1 potentiates G551D-CFTR-dependent Cl^- current in primary cultures of human nasal polyp epithelial (HNPE) cells. (A) Short-circuit current measured in HNPE cells from a CF patient with G551D/Y1092X mutations. G551D-CFTR was stimulated by application of 30 μM C_{act} -A1, 20 μM forskolin and 10 μM VX-770. G551D-CFTR-dependent current was inhibited by 20 μM $CFTR_{inh}$ -172. (B) Summary of peak currents (mean $\pm$ S.E., $n = 4-6$). * $P < 0.05$; ** $P < 0.001$.

Because C_{act} -A1 can activate CFTR in the absence of cAMP agonists, there are potential concerns about in vivo side effects, such as increased intestinal fluid secretion and diarrhea. However, in CF patients with loss of function CFTR mutations, inappropriate overactivation of CFTR by C_{act} -A1 is unlikely.

Current results suggest that drug therapy of CF caused by the $\Delta F508$ mutation will require a corrector to rescue $\Delta F508$ -CFTR cell surface expression, and a potentiator to increase its Cl^- conductance. Recent clinical data using the corrector VX-809 (3-[6-[[[1-(2,2-difluoro-1,3-benzodioxol-5-yl)cyclopropyl]carbonyl]amino]-3-methyl-2-pyridinyl]-benzoic acid) in CF patients with $\Delta F508$ mutation suggest that it will not be sufficient to produce a clinical benefit (Van Goor et al., 2011; Clancy et al., 2012). VX-809 is currently in phase 2 clinical trials using combination treatment with VX-770. The addition of a potentiator to maximize cell Cl^- conductance is the accepted current concept in CF therapeutics development. Alternatively, an efficient corrector or combination of correctors that restore proper $\Delta F508$ -CFTR folding and plasma membrane targeting may obviate the need for a potentiator. The combination of a corrector and an activator such as C_{act} -A1 here may be beneficial to maximize $\Delta F508$ -CFTR Cl^- conductance, even in the absence of cAMP elevation.

In summary, C_{act} -A1, a novel CFTR activator, activated CFTR Cl^- current without cAMP elevation and was CFTR-selective,

reversible, and nontoxic. In primary cultures of human airway epithelial cells, C_{act} -A1 strongly stimulated WT- and $\Delta F508$ -CFTR to the same level as that produced by forskolin, and showed additive activation of $\Delta F508$ -CFTR with forskolin. C_{act} -A1 may be useful for elucidating molecular mechanisms of CFTR activation and as a potential CF drug development candidate.

Authorship Contributions

Participated in research design: Namkung, Verkman.

Conducted experiments: Namkung, Park, Seo.

Contributed new reagents or analytic tools: Namkung.

Performed data analysis: Namkung.

Wrote or contributed to the writing of the manuscript: Namkung, Verkman.

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