Nanomolar Affinity Small Molecule Correctors of Defective ΔF508-CFTR Chloride Channel Gating

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Deletion of Phe-508 (ΔF508) is the most common mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) causing cystic fibrosis. ΔF508-CFTR has defects in both channel gating and endoplasmic reticulum-to-plasma membrane processing. We identified six novel classes of high affinity potentiators of defective ΔF508-CFTR Cl⁻ channel gating by screening 100,000 diverse small molecules. Compounds were added 15 min prior to assay of iodide uptake in epithelial cells co-expressing ΔF508-CFTR and a high sensitivity halide indicator (YFP-H148Q/F152L) in which ΔF508-CFTR was targeted to the plasma membrane by culture at 27 °C for 24 h. Thirty-two compounds with submicromolar activating potency were identified; most had tetrahydrobenzothiophene, benzofuran, pyrimidinetrione, dihydropyridine, and anthraquinone core structures (360–480 daltons). Further screening of >1000 structural analogs revealed tetrahydrobenzothiophenes that activated ΔF508-CFTR Cl⁻ conductance reversibly with Kᵢ < 100 nM. Single-cell voltage clamp analysis showed characteristic CFTR currents after ΔF508-CFTR activation. Activation required low concentrations of a cAMP agonist, thus mimicking the normal physiological response. A Bayesian computational model was developed using tetrahydrobenzothiophene structure-activity data, yielding insight into the physical character and structural features of active and inactive potentiators and successfully predicting the activity of structural analogs. Efficient potentiation of defective ΔF508-CFTR gating was also demonstrated in human bronchial epithelial cells from a ΔF508 cystic fibrosis subject after 27 °C temperature rescue. In conjunction with correctors of defective ΔF508-CFTR processing, small molecule potentiators of defective ΔF508-CFTR gating may be useful for therapy of cystic fibrosis caused by the ΔF508 mutation.

Cystic fibrosis (CF) is the most prevalent hereditary lethal disease in Caucasians. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a cAMP-activated Cl⁻ channel expressed in airway, intestinal, pancreatic, and other secretory and absorptive epithelia. The principal clinical problem in CF is recurrent lung infections that result in progressive deterioration in lung function. The most common CFTR mutation, deletion of Phe-508 (ΔF508-CFTR), is present in at least one allele in ~90% of CF patients (1). The ΔF508 mutation causes two distinct defects in CFTR that produce Cl⁻-impermeable cells: (i) retention at the endoplasmic reticulum caused by misfolding and/or defective interactions with molecular chaperones (2, 3), and (ii) impaired intrinsic Cl⁻ conductance (reduced open channel probability) (4–6).

Strategies have been discovered to correct the defects in ΔF508-CFTR cellular processing and Cl⁻ channel gating in cell culture models. Cell growth at low temperature (<30 °C) (2) or with high concentrations of chemical chaperones such as glycerol (0.5, 8) partially corrects the defective ΔF508-CFTR cellular processing by a mechanism that may involve improved protein folding and stability (9). A sustained increase in intracellular calcium concentration by thapsigargin also corrects defective ΔF508-CFTR processing (10), possibly by interfering with interactions with molecular chaperones. Compounds like phenylbutyrate facilitate ΔF508-CFTR cellular processing by altering chaperone function and/or transcriptional enhancement (11, 12).

ΔF508-CFTR has significantly impaired channel activity (“gating defect”) even when present at the cell plasma membrane (4). Cell-attached patch clamp measurements showed reduced ΔF508-CFTR open channel probability and prolonged closed times even with maximal cAMP stimulation (5, 6). Patch clamp measurements in excised membranes indicated a 7-fold reduced rate of activation of ΔF508-CFTR after phosphorylation compared with wild-type CFTR (13). Relatively high concentrations of the flavone genistein (>50 µM, Refs. 6 and 13) or the xanthine isobutylmethylxanthine (>1 mM, Ref. 14) in combination with cAMP agonists increase ΔF508-CFTR channel activity.

Previously, we screened a collection of 60,000 diverse small molecules for activation of wild-type CFTR (15). Although more

*This work was supported in part by a CF Drug Discovery Grant from the Cystic Fibrosis Foundation and National Institutes of Health Grants HL73856, EB00415, HL59198, EY13574, and DK35124. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by a National Defense Science and Engineering Fellowship.

‡Supported by the Sandler Research Foundation.

§Supported by the Canadian Cystic Fibrosis Foundation.

*Supported by Grant GP0296Y01 from Telethon-Italy.

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than a dozen classes of compounds were identified with submicromolar activating potency, most activators of wild-type CFTR did not activate ΔF508-CFTR, even after targeting to the cell surface and stimulation by high concentrations of cAMP agonists. Only two of the most potent activators of wild-type CFTR had near micromolar potency for ΔF508-CFTR activation. Given the apparent and quite different requirements for activation of wild-type versus ΔF508-CFTR, we postulated that there may exist novel classes of high affinity ΔF508-CFTR activators.

The purpose of this study was to identify and characterize compounds (termed “potentiators”) that correct defective ΔF508-CFTR gating. A stably transfected epithelial cell line co-expressing ΔF508-CFTR and a green fluorescent protein mutant with ultra-high halide sensitivity (YFP-H148Q/I52L, Ref. 16) was generated in which growth of cells at low temperature (27 °C) for 24 h gave consistent ΔF508-CFTR expression at the plasma membrane. A collection of 100,000 diverse drug-like small molecules was screened for activation of halide transport in these cells. We identified >30 compounds that corrected defective ΔF508-CFTR Cl− channel gating with submicromolar affinity, with most compounds belonging to six distinct chemical classes that are structurally unrelated to known CFTR activators or inhibitors. Tetrahydrobenzothio-phenes with ΔF508-CFTR-activating potencies down to 60 nm were identified and characterized. The compounds were shown to correct defective ΔF508-CFTR gating in low temperature-rescued human bronchial cells from a ΔF508 CF patient. Also, the hypothesis was tested that the ΔF508-CFTR potentiators could correct ΔF508-CFTR missplicing and retention at the endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Cell Lines—Clonal populations of Fischer rat thyroid (FRT) epithelial cells stably co-expressing human ΔF508-CFTR and the high sensitivity halide-sensing green fluorescent analog YFP-H148Q/I52L (16) were generated by liposome transfection and limiting dilution with Zeocin/G418 selection. More than 100 clones were evaluated for high fluorescence and ΔF508-CFTR plasma membrane targeting after growth at 27 °C for 24 h. For screening, cells were cultured on plastic in Coon’s modified F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and plated on black 96-well microplates (Corning-Costar 3904) at 30,000 cells/well. For short-circuit measurements, cells were cultured on Snapwell permeable supports (Corning-Costar) at 500,000 cells/well. Human bronchial epithelial cells homozygous for the ΔF508-CFTR mutation were cultured on Snapwell inserts and allowed to differentiate in a hormone-supplemented medium, as described previously (17). Some measurements were done with stably transfected FRT cells expressing YFP-H148Q and wild-type or G551D-CFTR (18). Patch clamp experiments were done on ΔF508-CFTR-expressing FRT cells plated on 35-mm Petri dishes.

Compounds—A collection of 100,000 diverse drug-like compounds (molecular sizes = 350–550 daltons, ChemBridge Co.) was used for initial screening. For optimization, >1000 analogs of activators identified in the primary screen were purchased from ChemBridge or ChemD (of ~600,000 available compounds) or synthesized and purified. Compounds were prepared as 10 μM stock solutions in Me2SO. Secondary plates containing one or four compounds/well were prepared for screening (0.25 μM in Me2SO). Compounds for secondary analysis were purified and confirmed by NMR and liquid chromatography/mass spectrometry.

Synthesis of Tetrahydrobenzothio-phenes—Compounds with different R1 substituents (see Fig. 3A) were synthesized by first preparing the 2-amino-tetrahydrobenzo[b]thiophene derivative by reaction of cyclohexane and sulfur with 2-cyanoacetamide in the presence of diethylamine (19). The product in pyridine was reacted with 2-chlorobenzoyl chloride in benzene, extracted in benzene, and recrystallized in ethyl acetate-hexane (yield 70–80%). Compound structures were confirmed by 1H NMR and mass spectrometry.

Screening Procedures—Screening was carried out using a Beckman integrated system containing a 3-meter robotic arm, CO2 incubator containing microplate carousel, plate washer, liquid handling work station, bar code reader, delidding station, plate reader, and two FLU- ostar fluorescence plate readers (Galaxy, BMG Lab Technologies), each equipped with dual syringe pumps and HSQ500/20X (500 ± 10 nm) excitation and HSQ535/30M (535 ± 15 nm) emission filters (Chroma) (details in Ref. 15). Software was written in Visual Basic for Applications version 6.3 to compute baseline-subtracted fluorescence slopes (giving halide influx rates).

A primary assay of ΔF508-CFTR potentiator activity, the incubator (27 °C, 90% humidity, 5% CO2/95% air) was loaded with 40–60 well plates containing FRT cells. After an 18- to 24-h incubation, plates were washed three times with PBS (300 μl/wash), leaving 50 μl of PBS. 10 μl of PBS containing 120 μM forskolin was added, and after 5 min, test compounds (0.6 μl of 0.25 mM Me2SO solution) were added to each well in final compound concentrations of 2.5 μM. The microplates were transferred to a plate reader for fluorescence assay. Each well was assayed individually for I− influx by recording fluorescence continuously (200 ms/point) for 2 s (baseline) and then for 12 s after the rapid (<1 s) addition of 160 μl of isomolar PBS in which 137 mM Cl− was replaced by I−. I− influx rates were computed from initial fluorescence versus time-curve slopes (determined by 3rd order polynomial regression) after normalization for total fluorescence (background-subtracted initial fluorescence).

Assays of cAMP and Phosphatase Activity—cAMP activity was measured with the BIOTRAK enzymatic immunoassay (Amer sham Biosciences) on FRT cell lysates after incubation with activators for 10 min without or with 0.5 μM forskolin. Phosphatase activity was determined on cell homogenates by using a nonradioactive assay kit (Promega), as described previously (18).

Short-circuit Current Measurements—Ussing chamber experiments were performed 7–9 days after plating ΔF508-CFTR-expressing FRT cells on Snapwell inserts. The basolateral solution contained (in mM): 120 NaCl, 2.7 KCl, 1.5 KH2PO4, 1 CaCl2, 0.5 MgCl2, 10 glucose, 10 Na-Hepes (pH 7.3). In the apical bathing solution, 65 mM NaCl was replaced by sodium gluconate, and CaCl2 was increased to 2 mM. Solutions were bubbled with air and maintained at 37 °C. The basolateral membrane was permeabilized with 250 μg/ml amphotericin B. For human bronchial epithelial cells, apical and basolateral chambers contained 130 mM NaCl, 38 mM KCl, 1.2 mM CaCl2, 2.4 mM NaHCO3, and 10 mM glucose (basolateral membrane not permeabilized). The hemichannels were connected to a DVC-1000 voltage clamp (World Precision Instruments) by means of Ag/AgCl electrodes and 1 KCl agar bridges for recording short-circuit current.

Whole-cell Patch Clamp—Cells were seeded at a density of 104 cells/well and used 2–4 days after plating. Borosilicate glass pipettes were fire-polished to obtain tip resistances of 2–4 megohms. Currents were sampled at 500 Hz using a patch clamp amplifier (EPC-7, List, Darmstadt) and low pass filtered using a 4-pole Bessel filter set at a cutoff frequency of 250 Hz. The extracellular (bath) solution contained (in mM): 150 NaCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 mannitol, and 10 TES (pH 7.4). The pipette solution contained (in mM): 120 CsCl, 1.5 MgCl2, 10 TEA-Cl, 0.5 EGTA, 1 Mg ATP, and 10 Hepes (pH 7.3). Membrane conductances were monitored by alternating the membrane potential between +80 and −100 mV. Current-voltage relationships were generated by applying voltage pulses between −100 and +100 mV in 20-mV steps.

Analysis of ΔF508-CFTR Missplicing—Cells were incubated at 37 °C in the presence of 10 μM ΔF508-CFTR potentiators. For functional studies, the plate reader assay was carried out 15 min after washing potentiators and adding forskolin (20 μM) and the potentiator ΔF508-CFTR −02 (2 μM). For biochemical analysis of ΔF508-CFTR glycosylation, baby hamster kidney cells expressing ΔF508-CFTR-β (hemagglutinin tagged, Ref. 9) were incubated with test compounds (10 μM) for 24 h at 37 °C. Cells were lysed in radioimmuno precipitation assay buffer, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-CFTR antibody mixture (M3A7 and L1B24 antibodies) or with anti-Na-K-ATPase antibody.

Computational Analysis—Data manipulations, property calculations, and model building were performed with PSC (Scitegic, Inc.). The data set for modeling consisted of 3025 tetrahydrobenzothio-phenes containing 40 active compounds. The Bayesian learning model contained the following parameters: molecular weight, surface area, polar surface area, number of H-bond donors, number of H-bond acceptors, AlogP, and the functional class fingerprint of Scitegic with a diversity of 6 bonds (δCFTR). The data set of tetrahydrobenzothio-phenes was partitioned randomly into four sets of approximately equal size. The Bayesian learner was trained on three of the four data partitions to distinguish between active and inactive tetrahydrobenzothio-phenes, producing four different models. Each Bayesian model re-
tered information from the inputted parameters into a single dimension, or “model score.” The Mann-Whitney test for nonparametric two-group comparisons was used to assess the likelihood that the distributions of model scores for active and inactive tetrahydrobenzothiophenes represent different populations. Favorable and unfavorable structural elements were extracted from the learning models using Pipeline Pilot’s Learned Property Viewer component. A congeneric series for structure-activity analysis was generated by removing the R group from each active compound and using the resulting scaffold to perform a substructure search for inactive tetrahydrobenzothiophenes.

RESULTS

Investigation of the plasma membrane ΔF508-CFTR gating defect and identification of potentiators required the development of cell lines and culture methods to reliably target ΔF508-CFTR to the plasma membrane. FRT cell clones were isolated that co-expressed human ΔF508-CFTR and a high sensitivity yellow fluorescent protein-based halide indicator. Cell lines were screened for ΔF508-CFTR plasma membrane function 18–36 h after growth at different temperatures and/or in the presence of agents reported previously to facilitate ΔF508-CFTR plasma membrane targeting (e.g., butyrate, glycerol, trimethylamine oxide). A cell clone was isolated that showed consistent ΔF508-CFTR plasma membrane targeting after 24 h of growth at 37 °C and had bright fluorescence, rapid growth on plastic support, and low basal halide leak. By short-circuit current analysis, strong ΔF508-CFTR Cl− currents were seen in transfected cells cultured at 27 °C but not at 37 °C (Fig. 1A). Notably, the plasma membrane-corrected ΔF508-CFTR had a significant gating defect, as seen from the submaximal activation by a high concentration of forskolin. Full activation required the addition of the known ΔF508-CFTR potentiator genistein. Other cell clones showed lower Cl− currents after 24 h of growth at 27 °C and no significant current when grown at 37 °C. To verify the gating defect in a native airway cell expressing ΔF508-CFTR, we were able to demonstrate consistent 27 °C rescue in human bronchial epithelial cells from a CF patient homozygous for the ΔF508-CFTR mutation (Fig. 1B). Full ΔF508-CFTR activation required genistein, as was found for the transfected cells, indicating the need for a potentiator after ΔF508-CFTR low temperature rescue.

A high throughput screen was designed to identify ΔF508-CFTR potentiators. FRT cells were incubated at 37 °C for 24 h to permit ΔF508-CFTR plasma membrane targeting (Fig. 2A). After washing, forskolin (20 μM) and test compounds (2.5 μM) were added to individual wells of 96-well plates. A high concentration of forskolin was used to identify ΔF508-CFTR potentiators that may interact directly with ΔF508-CFTR rather than alter cAMP concentration. The I− influx assay was carried out ~15 min later by measurement of the time course of decreasing YFP fluorescence after creation of an inwardly directed I− gradient.

Fig. 2B (top curve) shows representative time course data from a control well (“saline”) in which slow I− influx was seen when forskolin was added without test compounds. Examples of inactive compounds are shown. Each plate also contained positive control wells in which a dose-response was done for genistein, a known (though low potency) ΔF508-CFTR potentiator. Rapid I− influx was found for some of the 100,000 test compounds (Fig. 2B, bottom curves). Fig. 2C summarizes the results of the primary screen. Although most compounds had no significant ΔF508-CFTR potentiating activity at 2.5 μM, there were 75 strong (I− influx > 0.1 mV/s) and 252 weaker potentiators.

The strong potentiators were subjected to secondary analysis to select a subset for further analysis. None of the strong potentiators stimulated I− influx in the fluorescence assay using FRT-null cells (expressing YFP-H148Q/Y152L alone) or in ΔF508-CFTR-expressing cells in the absence of forskolin. The increased I− influx for each potentiator was blocked by the thiazolidinone CFTR inhibitor CFTRinh-172 (20). Dose-response studies were done to determine Kp and Vmax, with representative data shown in Fig. 2D. Of the 75 strong potentiators with >0.1 mV/s I− influx in the primary screen (at 2.5 μM), there were 32 compounds with Kp < 1 μM and Vmax greater than that of the reference compound genistein (at 50 μM).

Short-circuit current analysis was done on each of these compounds to confirm bona fide activation of ΔF508-CFTR Cl− currents. Experiments were done after basolateral membrane permeabilization and in the presence of a transepithelial Cl− gradient, so that short-circuit current represents apical membrane Cl− current. Representative data are shown in Fig. 3B. Thirteen compounds increased short-circuit current to levels comparable with that of maximal genistein but with Kp < 2 μM. None of the compounds activated short-circuit current in FRT-null cells or in ΔF508-CFTR-expressing FRT cells in the absence of forskolin. Most of the strong potentiators of ΔF508-CFTR Cl− conductance belonged to six distinct structural classes, with the chemical structures of the most potent compound of each class shown in Fig. 3A. A compound similar to class “O3” potentiators was identified in our previous screening for activators of wild-type CFTR (15), whereas the other compounds represent novel scaffolds. Interestingly, four of the compounds producing strong halide influx in the fluorescence assay did not produce Cl− currents by short-circuit current analysis (not shown), suggesting that they may induce electro-neutral halide transport through ΔF508-CFTR.

Whole-cell patch clamp was done to characterize the channels activated by ΔF508-CFTR potentiators. Fig. 3C (top) shows membrane currents after forskolin alone and then forskolin with genistein, demonstrating again the gating defect. After genistein washout, a ΔF508-CFTR potentiator gave similar membrane current. Current-voltage relationships generated in the presence of genistein or ΔF508-CFTR potentiators had the same linear ohmic behavior (Fig. 3C, bottom) as that found for activated wild-type CFTR. The currents showed no relaxation phenomena at positive or negative membrane potentials, thus providing evidence against the involvement of volume-sensitive or Ca2+-activated Cl− channels.

The six ΔF508-CFTR potentiators shown in Fig. 3A were tested for activation of wild-type and G551D-CFTR in transfected FRT cells. None of the compounds gave measurable G551D-CFTR activation at 10 μM in the presence of 20 μM...
forskolin, whereas strong activation was found for the positive control (50 \( \mu \)M genistein + 20 \( \mu \)M forskolin). All \( \Delta F508 \)-CFTR potentiators activated wild-type CFTR, but only in the presence of a low concentration of forskolin (50 \( nM \)), which did not itself activate CFTR. \( K_d \) values for activation of wild-type CFTR by \( \Delta F508_{\text{act}} \)-01 through \( \Delta F508_{\text{act}} \)-06 were (in \( nM \)): 0.18 \( \pm \) 0.02, 1.3 \( \pm \) 0.2, 2.2 \( \pm \) 0.3, 0.02 \( \pm \) 0.005, 0.06 \( \pm \) 0.01, and 0.05 \( \pm \) 0.01, respectively. These potencies are quite different from those for \( \Delta F508 \)-CFTR activation. For comparison, \( K_d \) values for the activation of \( \Delta F508 \)-CFTR by \( \Delta F508_{\text{act}} \)-01 through \( \Delta F508_{\text{act}} \)-06 from the fluorescence assay were (in \( nM \)): 1.3 \( \pm \) 0.1, 0.18 \( \pm \) 0.03, 0.70 \( \pm \) 0.04, 0.87 \( \pm \) 0.1, 0.10 \( \pm \) 0.01, and 0.65 \( \pm \) 0.08, respectively.

A secondary library of >1000 compounds with structural similarity to each class of \( \Delta F508 \)-CFTR potentiators was screened to establish structure-activity relationships and to identify the best compounds for further analysis. Structural analogs of the benzofuran, pyrimidinetrione, dihydroxypridine, and anthraquinone classes with good \( \Delta F508 \)-CFTR-activating potencies were not identified. However, 17 tetrahydrobenzothiophene analogs (class 02) were identified as giving good \( \Delta F508 \)-CFTR activation. The \( K_d \) and \( V_{\text{max}} \) of the six strongest \( \Delta F508 \)-CFTR potentiators are summarized in Fig. 4A. Fig. 4B shows our procedure for the synthesis of tetrahydrobenzothiophene analogs.

Further analysis showed rapid \( \Delta F508 \)-CFTR activation (Fig. 5A, left), with half-maximal activation in <3 min. Activation was fully reversed for most of the compounds at 60 min after washout (Fig. 5A, right). \( \Delta F508 \)-CFTR activation required low concentrations of forskolin (Fig. 5B). Fig. 5C shows that the tetrahydrobenzothiophenes induced strong Cl\(^{-}\) currents in short-circuit experiments with submicromolar activating potencies, both in temperature-rescued \( \Delta F508 \)-CFTR-expressing FRT cells (left) and human bronchial epithelial cells (right). The mean increase in short-circuit current (\( I_{sc} \)) was 1.2 \( \pm \) 0.1 \( \mu \)A/cm\(^2\) in the human cells (S.E., \( n = 25 \)). In five sets of measurements on the human bronchial cells, the percentage increase in \( I_{sc} \) after compound versus forskolin alone was 174 \( \pm \) 28 (genistein); percentages for \( \Delta F508_{\text{act}} \)-01 through \( \Delta F508_{\text{act}} \)-06 were (S.E., \( n = 3-5 \)): 174 \( \pm \) 34, 131 \( \pm \) 35, 40 \( \pm \) 11, 51 \( \pm \) 17, 107 \( \pm \) 42, and 104 \( \pm \) 35, respectively.

The \( \Delta F508 \)-CFTR potentiators (shown in Figs. 3A and 4A) were assayed for cAMP stimulation and phosphatase inhibition. Cellular cAMP content was measured in FRT cells in the presence of a low forskolin concentration (0.5 \( \mu \)M), with or without test compounds. As positive controls, a phosphodiesterase inhibitor (isobutylmethylxanthine, 50 \( \mu \)M) and a cAMP-elevating CFTR activator (CFTR-act-16, 5 \( \mu \)M; Ref. 15) strongly increased cAMP content from 129 \( \pm \) 7 to 1110 \( \pm \) 56 and 1733 \( \pm \) 51 fmol/well, respectively. Maximal forskolin (20 \( \mu \)M) gave 1350 \( \pm \) 17 fmol/well. The \( \Delta F508 \)-CFTR potentiators at 5 \( \mu \)M gave no increase in cellular cAMP content, except for \( \Delta F508_{\text{act}} \)-04 and \( \Delta F508_{\text{act}} \)-06, which gave modest cAMP elevations (212 \( \pm \) 17 and 281 \( \pm \) 37 fmol/well, respectively). Phosphatase assay showed no inhibition of phosphatase activity by the \( \Delta F508 \)-CFTR potentiators under conditions where the known phosphatase inhibitor okadaic acid inhibited phosphatase activity by >90% (from 703 \( \pm \) 69 to 56 \( \pm \) 15 pmol of free phosphate per \( \mu \)g of protein). The \( \Delta F508 \)-CFTR potentiators (25 \( \mu \)M, 48 h) were judged to be non-toxic to FRT cells by the dihydrorhodamine assay (13) and by unimpaired cell growth.

Because the \( \Delta F508 \)-CFTR potentiators probably activate plasma membrane-targeted \( \Delta F508 \)-CFTR by a direct interaction mechanism, we tested whether these compounds might correct \( \Delta F508 \)-CFTR cellular misprocessing (retention at endoplasmic reticulum). \( \Delta F508 \)-CFTR-expressing cells were incubated for 24 h at 37 °C with the potentiators (10 \( \mu \)M). Plasma membrane \( \Delta F508 \)-CFTR was assessed biochemically and functionally. Fig. 6A shows core- and complex-glycosylated forms for wild-type CFTR and for \( \Delta F508 \)-CFTR after a 26 °C rescue in Chinese hamster ovary cells. Little or no complex-glycosylated \( \Delta F508 \)-CFTR (C-band) was found after incubation of cells with the potentiators for 24 h at 37 °C. Similar results were obtained on \( \Delta F508 \)-CFTR-expressing FRT cells (not shown). For functional assay, cells were washed after 24 h, and \( \Gamma \) influx was measured 15 min after the addition of forskolin (20 \( \mu \)M) and the strong potentiator \( \Delta F508_{\text{act}} \)-02 (2 \( \mu \)M). Fig. 6B shows little increase in the rate of \( \Gamma \) influx (\( |d[\Gamma]/dt| \)) by the potentiators, with positive 27 °C rescue control. As a first step in lead optimization, a computational model relating transport activity to structural and physico-chemical parameters of the tetrahydrobenzothiophene class of \( \Delta F508 \)-
CFTR potentiators was generated by using a Bayesian learning methodology. The extracted minimal consensus substructure and physical properties of active tetrahydrobenzothiophenes are shown in Fig. 7A. The substructure allows for variation in the composition of the ring fused to the tetrahydrobenzothiophene and the group appended to the nitrogen at the 2-position of the tetrahydrobenzothiophene, but requires an amide at the 3-position and an amide or weakly basic group at the 2-position. The physical properties of the active subset of tetrahydrobenzothiophenes clearly differed from those of the full set of tetrahydrobenzothiophenes in the screening library (AlogP distribution shown in Fig. 7B). They also represent a distinct subset of the classic Lipinski parameters (21). The number of hydrogen bond donors and acceptors was low (<2 each), and the overall polar surface (72 ± A2 ± 98) and AlogP (2.3–3.6) fell within a narrow range. The learning model was successfully trained to distinguish between active and inactive tetrahydrobenzothiophenes and was cross-validated (four data partitions, p < 0.00001, regardless of originating training set) (Fig. 7C).

Further analysis of structure-activity trends was carried out by extracting the fingerprints from the active and inactive sets in the learned model, partitioning them into congeneric series, and examining the trends. Fig. 7D shows favorable and unfavorable structural elements identified by the Bayesian learning model from an analysis of Scitegic functional class fingerprints.
The purpose of this investigation was to identify high affinity small molecule potentiators of ΔF508-CFTR Cl⁻ conductance. A collection of 100,000 chemically diverse compounds was screened with a cell-based assay to detect ΔF508-CFTR-mediated halide influx. Incubation of ΔF508-CFTR-transfected cells for 24 h at 27 °C gave strong ΔF508-CFTR expression at the cell surface as needed for screening of rapidly acting potentiators of ΔF508-CFTR function. More than 30 ΔF508-CFTR potentiators were identified by the initial cell-based fluorescence screen with apparent submicromolar activating potencies. Electrophysiological analysis confirmed strong ΔF508-CFTR activating potency for most of the compounds, which fell in six structural classes. Optimization was done by screening >1000 structural analogs of the six classes. A series of tetrahydrobenzothiophenes was identified that activated ΔF508-CFTR Cl⁻ conductance reversibly with \( K_d \) down to 60 nM. A structure-activity series for the tetrahydrobenzothiophenes was generated for further optimization and computational modeling.

Functional analysis of ΔF508-CFTR potentiators indicated that they did not induce Cl⁻ currents in the absence of CFTR and that Cl⁻ currents in ΔF508-CFTR-expressing cells required cAMP and were inhibited by the thiazolidinone CFTRinh-172. The potentiators were rapidly acting, reversible, and non-toxic. Whole-cell patch clamp experiments showed that the activated currents were as expected for CFTR but not for other types of epithelial Cl⁻ channels. The potentiators did not induce currents in the absence of CFTR and that Cl⁻ currents in ΔF508-CFTR-expressing cells required cAMP and were inhibited by the thiazolidinone CFTRinh-172. The potentiators were rapidly acting, reversible, and non-toxic. Whole-cell patch clamp experiments showed that the activated currents were as expected for CFTR but not for other types of epithelial Cl⁻ channels. The potentiators did
not elevate cellular cAMP or inhibit cellular phosphatase activity. Thus, the potentiators probably activate ∆F508-CFTR Cl⁻ conductance by a direct interaction mechanism rather than affecting upstream signaling pathways or ∆F508-CFTR cellular trafficking. Interestingly, the ∆F508-CFTR potentiators also activated wild-type CFTR but did so with different relative potencies than for the activation of ∆F508-CFTR. None of the compounds activated G551D-CFTR even in the presence of a high concentration of a cAMP agonist nor did they cause endoplasmic reticulum-to-plasma membrane transport of ∆F508-CFTR, as assessed functionally and biochemically.

Analysis of the physical and structural determinants of the tetrahydrobenzothiophene ∆F508-CFTR potentiators using Bayesian computational methods revealed that they represent a statistically distinct subset of all tetrahydrobenzothiophenes in the screening library. The model effectively predicted activities of tetrahydrobenzothiophenes in cross-validation experiments. In an initial test of the general validity of this model, a series of ~135 previously untested tetrahydrobenzothiophenes was selected from a commercial source by using simple similarity comparisons. The Bayesian model correctly predicted the activities of three of the three most active compounds and the inactivity of ~90% of the inactive compounds. This model is applicable in guiding the synthesis of new compounds to establish well populated congenic series for quantitative structure-activity relationship analysis, as well as for virtual screening of commercially available compound collections.

In summary, high affinity ∆F508-CFTR potentiators were identified by high throughput screening of a diverse small molecule collection. The potentiators might activate ∆F508-CFTR by direct binding to a site on the first nucleotide-binding domain of CFTR, where the ∆F508 mutation site is located. The compounds may be useful in elucidating the ∆F508-CFTR folding defect and for co-crystallization with ∆F508-containing CFTR domains. The potentiators may have clinical utility as ∆F508-CFTR activators when used in conjunction with compounds or maneuvers that correct defective ∆F508-CFTR cellular misprocessing and possibly when used alone in a subset of ∆F508 CF patients having a mild form of the disease with partial ∆F508-CFTR plasma membrane expression.

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