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/I152L) 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, pyramidinetrione, dihydropyridine, and anthraquinone core structures (360–480 daltons). Further screening of >1000 structural analogs revealed tetrahydrobenzothiophenes that activated ΔF508-CFTR Cl− conductance reversibly with \( K_c < 100 \) 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 glyceral (7, 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 isobutylmethanxthine (>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

The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FRT, Fischer rat thyroid; HA, hemagglutinin; YFP, yellow fluorescent protein; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; PBS, phosphate-buffered saline; TEA, tetraethylammonium.
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/I152L, 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. Tetrahydrobenzothiophenes 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 misprocessing 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/I152L (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 t-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. For short-circuit measurements, cells were incubated at 30,000 cells/well. For optimization, cells were cultured by applying voltage pulses between +100 mV. Current-voltage relationships were generated by applying voltage pulses between +100 and −100 mV in 20-mV steps.

Analysis of ΔF508-CFTR Misprocessing—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 glycosylation, baby hamster kidney cells expressing ΔF508-CFTR-HA (hemagglutinin-tagged, Ref. 9) were incubated with test compounds (10 µM) for 24 h at 37 °C. Cells were lysed in radioimmune precipitation assay buffer, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-CFTR antibody mixture (M3A7 and L12B4 antibodies) or with anti-NaK-ATPase antibody.

Computational Analysis—Data manipulations, property calculations, and model building were performed with PDB (Scitegic, Inc.). The data set for modeling consisted of 3025 tetrahydrobenzothiophenes 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 database of 6 bonds (PFC) (details in Ref. 15). 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 tetrahydrobenzothiophenes, producing four different models. Each Bayesian model re-
Transfected FRT cells

Human bronchial cells

Fig. 1. Low temperature correction of ΔF508-CFTR misprocessing in transfected FRT cells (A) and primary cultures of human bronchial epithelium from a homozygous CF patient (B).

Transepithelial short-circuit current (Isc) was measured (at 37 °C) after a 24-h incubation at 37 °C (top) or 27 °C (bottom). Where indicated, forskolin (20 µM), CFTRinh-172 (10 µM), amiloride (100 µM), or indicated concentrations of genistein were added.

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 CFTR to the plasma membrane. FRT cell clones were isolated by genetic selection, or produced 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 tetrahydrobenzo-thiophenes 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 tetrahydrobenzo-thiophenes.

Forskolin (20 µM) at 37 °C 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 mA/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/V152L 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 Kd and Vmax with representative data shown in Fig. 2D. Of the 75 strong potentiators with >0.1 mA/s I− influx in the primary screen (at 2.5 µM), there were 32 compounds with Kd < 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 Kd < 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 “03” 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 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-sensi- tive 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 μM genistein + 20 μM forskolin). All ΔF508-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. KC values for activation of wild-type CFTR by ΔF508-act-01 through ΔF508-act-06 were (in μM): 0.18 ± 0.02, 1.3 ± 0.2, 2.2 ± 0.3, 0.02 ± 0.005, 0.06 ± 0.01, and 0.05 ± 0.01, respectively. These potencies are quite different from those for ΔF508-CFTR activation. For comparison, KC values for the activation of ΔF508-CFTR by ΔF508-act-01 through ΔF508-act-06 from the fluorescence assay were (in μM): 1.3 ± 0.1, 0.18 ± 0.03, 0.70 ± 0.04, 0.87 ± 0.1, 0.10 ± 0.01, and 0.65 ± 0.08, respectively.

A secondary library of >1000 compounds with structural similarity to each class of ΔF508-CFTR potentiators was screened to establish structure-activity relationships and to identify the best compounds for further analysis. Structural analogs of the benzofuran, pyrindimetrione, dihydropyridine, and anthroquinone classes with good ΔF508-CFTR-activating potencies were not identified. However, 17 tetrahydrobenzothiophenes (class 02) were identified as giving good ΔF508-CFTR activation. The Kc and Vmax of the six strongest ΔF508-CFTR potentiators are summarized in Fig. 4A. Fig. 4B shows our procedure for the synthesis of tetrahydrobenzothiophene analogs.

Further analysis showed rapid ΔF508-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). ΔF508-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 ΔF508-CFTR-expressing FRT cells (left) and human bronchial epithelial cells (right). The mean increase in short-circuit current (Imax) was 1.2 ± 0.1 μA/cm² in the human cells (S.E., n = 25). In five sets of measurements on the human bronchial cells, the percentage increase in Imax after compound versus forskolin alone was 174 ± 28 (genistein); percentages for ΔF508-act-01 through ΔF508-act-06 were (S.E., n = 3–5): 174 ± 34, 131 ± 35, 40 ± 11, 51 ± 17, 107 ± 42, and 104 ± 35, respectively.

The ΔF508 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 μM), with or without test compounds. As positive controls, a phosphodiesterase inhibitor (isobutylmethylxanthine, 50 μM) and a CAMP- elevating CFTR activator (CFTR act-16, 5 μM; Ref. 15) strongly increased CAMP content from 129 ± 7 to 1110 ± 56 and 1733 ± 51 fmol/well, respectively. Maximal forskolin (20 μM) gave 1350 ± 17 fmol/well. The ΔF508-CFTR potentiators at 5 μM gave no increase in cellular cAMP content, except for ΔF508-act-04 and ΔF508-act-06, which gave modest CAMP elevations (212 ± 27 and 281 ± 37 fmol/well, respectively). Phosphatase assay showed no inhibition of phosphatase activity by the ΔF508 potentiators under conditions where the known phosphatase inhibitor okadaic acid inhibited phosphatase activity by >90% (from 703 ± 69 to 56 ± 15 pmol of free phosphate per μg of protein). The ΔF508-CFTR potentiators (25 μM, 48 h) were judged to be non-toxic to FRT cells by the dihydrorhodamine assay (13) and by unimpaired cell growth.

Because the ΔF508-CFTR potentiators probably activate plasma membrane-targeted ΔF508-CFTR by a direct interaction mechanism, we tested whether these compounds might correct ΔF508-CFTR cellular misprocessing (retention at endoplasmic reticulum). ΔF508-CFTR-expressing cells were incubated for 24 h at 37 °C with the potentiators (10 μM). Plasma membrane ΔF508-CFTR was assessed biochemically and functionally. Fig. 6A shows core- and complex-glycosylated forms for wild-type CFTR and for ΔF508-CFTR after a 26 °C rescue in Chinese hamster ovary cells. Little or no complex-glycosylated ΔF508-CFTR (C-band) was found after incubation of cells with the potentiators for 24 h at 37 °C. Similar results were obtained on ΔF508-CFTR-expressing FRT cells (not shown). For functional assay, cells were washed after 24 h, and I− influx was measured 15 min after the addition of forskolin (20 μM) and the strong potentiator ΔF508-act-02 (2 μM). Fig. 6B shows little increase in the rate of I− influx (Δd[I−]/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 ΔF508-
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 (≤3 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.
Fig. 7 illustrates a structure-activity series derived from the screening data. The seminal structural features of the model include: (i) presence of a 4,5-fused tetrahydrobenzothiophene, with the fused ring being a 6- or 7-membered aliphatic ring; (ii) presence of an unsubstituted carboxamide in the 3-position; and (iii) a high population of aromatic amides at the 2-position.

**DISCUSSION**

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<sub>d</sub> 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
High Affinity ΔF508-CFTR Potentiators

not elevate cellular cAMP or inhibit cellular phosphatase activity. Thus, the potentiators probably activate ΔF508-CFTR Cl⁻ conductance by a direct interaction mechanism rather than affecting upstream signaling pathways or ΔF508-CFTR cellular trafficking. Interestingly, the ΔF508-CFTR potentiators also activated wild-type CFTR but did so with different relative potencies than for the activation of ΔF508-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 ΔF508-CFTR, as assessed functionally and biochemically.

Analysis of the physical and structural determinants of the tetrahydrobenzothiophene ΔF508-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 congeneric series for quantitative structure-activity relationship analysis, as well as for virtual screening of commercially available compound collections.

In summary, high affinity ΔF508-CFTR potentiators were identified by high throughput screening of a diverse small molecule collection. The potentiators might activate ΔF508-CFTR by direct binding to a site on the first nucleotide-binding domain of CFTR, where the ΔF508 mutation site is located. The compounds may be useful in elucidating the ΔF508-CFTR folding defect and for co-crystallization with ΔF508-containing CFTR domains. The potentiators may have clinical utility as ΔF508-CFTR activators when used in conjunction with compounds or maneuvers that correct defective ΔF508-CFTR cellular misprocessing and possibly when used alone in a subset of ΔF508 CF patients having a mild form of the disease with partial ΔF508-CFTR plasma membrane expression.

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