High-affinity Activators of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Chloride Conductance Identified by High-throughput Screening*

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Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator protein (CFTR). This protein is a cAMP-activated anion channel expressed in mammalian airways and other epithelia. The purpose of this investigation was to identify new classes of potent CFTR activators. A collection of 60,000 diverse drug-like compounds was screened at 10 μM together with a low concentration (0.5 μM) of forskolin in Fisher rat thyroid epithelial cells co-expressing human CFTR and a green fluorescent protein-based Cl⁻ sensor. Primary screening yielded 57 strong activators (greater activity than reference compound apigenin), most of which were unrelated in chemical structure to known CFTR activators, and 284 weaker activators. Secondary analysis of the strong activators included analysis of CFTR specificity, forskolin requirement, transepithelial short-circuit current, activation kinetics, dose response, toxicity, and activation mechanism. Three compounds, the most potent being a dihydroisouquinoline, activated CFTR by elevating cellular cAMP, probably by phosphodiesterase inhibition. Fourteen compounds activated CFTR without cAMP elevation or phosphatase inhibition, suggesting direct CFTR interaction. The most potent compounds had tetrahydrocarbazol, hydroxycoumarin, and thiazolidine core structures. These compounds induced CFTR Cl⁻ currents rapidly (<5 min) with Kᵅ down to 200 nM and were CFTR-selective, reversible, and nontoxic. Several compounds, the most potent being a trifluoromethylphenylbenzamine, activated the CF-causing mutant G551D, but with much weaker affinity (Kᵅ > 10 μM). When added for 10 min, none of the compounds activated ΔPhe⁵⁰⁸-CFTR in transfected cells grown at 37 °C (with ΔPhe⁵⁰⁸-CFTR trapped in the endoplasmic reticulum). However, after correction of trafficking by 48 h of growth at 27 °C, tetrahydrocarbazol and N-phenyltriazine derivatives strongly stimulated Cl⁻ conductance with Kᵅ < 1 μM. The new activators identified here may be useful in defining molecular mechanisms of CFTR activation and as lead compounds in CF drug development.

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1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator protein; CF, cystic fibrosis; PDZ, PSD-95, Disc-large, and ZO-1; FRT, Fischer rat thyroid; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline.
instrumentation was set up, and procedures were established for rapid quantitative screening of individual compounds in a 96-well plate format. The primary screen was designed to identify CFTR activators that are effective without and in synergy with cAMP activators, the latter mimicking the normal physiological response. The primary screen identified more than 50 strong CFTR activators, most of which were unrelated chemically to known CFTR activators. The activators were subjected to extensive secondary analysis. Novel activators were identified that strongly activated CFTR Cl⁻ conductance at concentrations well under 1 μM and without elevation of cAMP, as well as compounds that activated the CF-causing mutants G551D-CFTR and ΔPhe⁵⁰⁵⁶⁶-CFTR.

MATERIALS AND METHODS

Cell Culture—Fischer rat thyroid (FRTL) cells stably co-expressing human CFTR (wild-type, or mutants G551D or ΔPhe⁵⁰⁵⁶⁶) and the yellow fluorescent protein YFP-H148Q were cultured on plastic in Coon’s modified F12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin as described previously (13). For fluorescence assays, cells were plated using a LabSystems multispot dispenser into black 96-well microplates with clear plastic bottoms (Corning-Costar 3904) at 30,000 cells/well. For Ussing chamber experiments, FRTL cells were transferred to Snapwell permeable supports (Corning-Costar) at 500,000 cells/well. Human bronchial epithelial cells were cultured on plastic as described previously (16). For short-circuit current measurements, the cells were plated on Snapwell inserts (500,000 cells/insert) and allowed to differentiate in a hormone-supplemented medium (16).

Compounds—Maximally diverse compound collections were obtained as 10 mM stock solutions in Me₃SO from ChemBridge (50,000 compounds) and ChemDiv (10,000 compounds) (both in San Diego, CA). The compounds were drug-like with average molecular mass of ~450 daltons, good Me₃SO and water solubility, and purity >95%. Screenable plates for screening (1 mM concentrations) were prepared by 10-fold dilutions with Me₃SO.

Instrumentation—Screening was carried out on a customized apparatus (SAGIAN, Beckman) containing a 3-m rail Optimized Robot for Chemical Analysis that integrates the following robot-accessible instruments on a 4 X 2-m optical table: (a) SAGIAN 180 CO₂ incubator (Perma SCientific), which holds 180 standard microplates in a storage carousel; (b) Elx405-Select plate washer (Bio-Tek Instruments) with valve option and vacuum-sensing/waste alert for complex automated wash cycles; (c) SAGIAN MPS-8 CS incubator (holds eight standard microplates at 4–37 °C); (d) SAGIAN shaker (six plate positions, frequency range 40–1100 rpm with variable amplitude); (e) Biomek 2000 liquid handling work station (installed with four positions to hold tip boxes with automatic air locks and six positions for microplates and liquid reservoirs, and MP 20 and MP 200 8-channel tip tools for liquid transfer); (f) SAGIAN carousel (holding 40 pipette tip boxes and 90 microplates); (g) SAGIAN bar code reader; (h) SAGIAN microplate lidding station with automatic vacuum control of six suction cups; (i) two Fluorstar fluorescence plate readers (BMG Lab Technologies), each equipped with two-syringe pumps and HQ555/20X (500 ± 10 nm) excitation and HQ555/20M (555 ± 15 nm) emission filters (Chroma). The system was integrated and automated using SAMI version 3.3 software (Beckman Coulter) (Fig. 1A).

Screening Procedures—To set up the assay, microplates containing cultured cells were loaded into a carousel in the CO₂ incubator set at 37 °C, 90% humidity, 5% CO₂. Compound plates (bar-coded) containing 1 mM test compound in Me₃SO in each well were loaded on 18-shelf carousel in the carousel. Each compound plate contained 80 different compounds, with the first and last columns used for negative (Me₃SO alone) and positive (1–25 mM apigenin) controls, respectively. MP20 tip boxes (E & K Scientific) were loaded on eight-carrier holders in the carousel. In each assay cycle, the robot retrieved an assay plate from the CO₂ incubator, and each well was washed four times in the plate washer by repeated dispensing and aspirating 200 μl of PBS, leaving 50 μl of PBS after the final aspiration. The assay plate was then incubated for 15 min at 37 °C in the SAGIAN MPS-8 CO₂ incubator. During the incubation, the robot transported a compound plate and pipette tip box to the liquid handling work station. After the 15-min incubation, the plate containing cells was transported to the liquid handling work station and 0.5 μl of each compound (to give final 10 μM compound concentration) was added to each well with two rounds of side mixing. The plate was then shaken for 30 s at 200 rpm and placed in one of the two fluorescence plate readers. Each assay consisted of a continuous 14-s read (5 points/s) with 2 s before solution addition and 12 s after injection of 165 μl of an I⁻–containing solution (PBS with 100 mM I⁻ replaced by I⁻). At 22 s before each fluorescence reading, 10 μl of PBS containing forskolin (0.5 μM final concentration) was added. After assays on a full plate were completed, data were stored and the plate was immediately disassembled and read on the other plate reader. All operations were integrated so that both plate readers were occupied >80% of the time as transport, incubation, and liquid transfer operations took place simultaneously.

Data Analysis—Custom software was developed for analysis of fluorescence data from the plate readers. The software was developed using Microsoft Visual Basic for Applications and was integrated with Microsoft Excel software. The calculated results were entered into a relational database (Microsoft Access) that managed data for each of the compounds for efficient analysis and tabulation. For each data curve, initial fluorescence was determined by averaging 10 time points (over 10 s) before I⁻ solution addition and subtracting background (generally <10%). A third-order polynomial was fitted to the final 11.5 s of the data and used to extrapolate the initial slope (just after I⁻ addition). Fluorescence slopes normalized for initial fluorescence (proportional to dF/dt) were computed and stored in the data base together with identification codes (from bar code reader).

Assays of cAMP, Phosphatase Activity, and Cell Toxicity—cAMP activity was measured using the BIOTRACK enzymatic immunoassay (Amersham Biosciences). FRTL cells expressing CFTR were cultured in 96-well plates. After washing and incubation in PBS, cells were incubated for 10 min with compounds and then lysed. Lynsates were assayed for cAMP content in triplicate according to the manufacturer’s instructions. Phosphatase activity was determined using a nonradioactive assay kit (Promega) as described previously (13). Briefly, an homogenate of CFTR-expressing FRTL cells (5 μg of protein per reaction) was incubated with the phosphomolybdate peptide substrate provided in the kit for 30 min at 37 °C. Released phosphate was measured colorimetrically at 620 nm. Cell toxicity was assayed by the sulforhodamine B method (15).
RESULTS

The primary screen of 60,000 diverse compounds was designed to identify compounds that activated CFTR when added alone as well as compounds that were required mild elevation of cytosolic cAMP. As depicted in Fig. 1B, individual test compounds at 10 μM concentration were added to one well of a 96-well plate 15 min before assay, and forskolin (0.5 μM) was added 22 s before assay. Each assay consisted of recording baseline fluorescence for 2 s followed by rapid addition of an I−-containing solution to drive I− entry into cells. The time course of decreasing YFP fluorescence was monitored over 12 s to give quantitative information about I− influx rates. The low concentration of forskolin produced little activation of CFTR by itself (<10% of maximal activity measured at 10 μM forskolin).

Fig. 2A shows results from a representative 96-well plate. The first column of wells in each plate contained negative and positive controls in which different concentrations of the reference activator apigenin were added. The remaining wells contained test compounds. The plate shown in Fig. 2A contained two wells (indicated by asterisks) in which there was increased I− entry. Fig. 2B shows original fluorescence curves for the apigenin dose response, along with representative inactive compounds, moderate activators, and strong activators.

Fig. 2C summarizes the rates of I− entry for the 60,000 test compounds. For reference, the averaged rates of I− entry were 0.01, 0.021, 0.19, and 1.0 mV/s for apigenin concentrations of 0, 1, 5, and 25 μM, respectively. The vast majority of compounds (59,659 of 60,000) had no significant effect (< 0.08 mV/s) on the rate of I− entry. Fifty-seven compounds, referred to as "weak activators," increased I− entry to >0.5 mV/s, and 284 compounds, referred to as "weak activators," increased I− entry to 0.08–0.5 mV/s. The strong activators were further analyzed, initially for CFTR specificity. Repeat fluorescence assay on all 57 strong activators (at 10 μM) confirmed the initial data. None of the strong activators increased I− entry in the fluorescence assay performed using CFTR null cells (FRT cells transfected with YFP-H148Q alone). As shown in Fig. 2D, some of the strong activators stimulated CFTR-dependent I− entry only in the presence of a small concentration of forskolin (CFTRact-11 shown as one example; others are CFTRact-01, -05, -07, and -10, whereas others (e.g., CFTRact-16; others are CFTRact-02, -03, -04, -06, -08, -09, -13, and -15) required forskolin for only maximal activation (see Fig. 3 for compound structures).
Transepithelial \( \text{Cl}^- \) current was measured in CFTR-expressing FRT cells grown on porous supports to select a subset of the 57 strong activators identified by the fluorescence assay that strongly increased \( \text{Cl}^- \) conductance at 1 mM. Each compound was tested at 1 and 2 mM concentrations after addition of 0.5 mM forskolin, which itself produced a small increase in short-circuit current. Fig. 3A shows maximal short-circuit current stimulated by 20 mM forskolin (left curve) and the much smaller current produced by 0.5 mM forskolin and 1–5 mM apigenin (second curve). Under these conditions, 17 of the compounds gave strong CFTR \( \text{Cl}^- \) currents (75 \( \mu \text{A/cm}^2 \) at 2 mM) and were selected for further characterization (representative data shown for CFTR act-05, CFTR act-07, and CFTR act-09). In each case, the increase in short-circuit current was inhibited by the CFTR blocker glibenclamide (glib) (400 \( \mu \text{M} \)) was added. B, chemical structures of the 17 most potent CFTR activators.

Transepithelial \( \text{Cl}^- \) current was measured in CFTR-expressing FRT cells grown on porous supports to select a subset of the 57 strong activators identified by the fluorescence assay that strongly increased \( \text{Cl}^- \) conductance at 1 \( \mu \text{M} \). Each compound was tested at 1 and 2 \( \mu \text{M} \) concentrations after addition of 0.5 \( \mu \text{M} \) forskolin, which itself produced a small increase in short-circuit current.

Fig. 3A shows maximal short-circuit current stimulated by 20 \( \mu \text{M} \) forskolin (left curve) and the much smaller current produced by 0.5 \( \mu \text{M} \) forskolin and 1–5 \( \mu \text{M} \) apigenin (second curve). Under these conditions, 17 of the compounds gave strong CFTR \( \text{Cl}^- \) currents (75 \( \mu \text{A/cm}^2 \) at 2 \( \mu \text{M} \)) and were selected for further characterization (representative data shown for CFTR act-05, CFTR act-07, and CFTR act-09). In each case, the increase in short-circuit current was inhibited by the CFTR blocker glibenclamide. Fig. 3B shows the chemical structures of these 17 compounds. Compound CFTR act-01 has a xanthine core structure, whereas the core structures of the other 16 compounds are unrelated to those of known CFTR activators. The structures of many of the remaining 30 strong activators and 284 weaker activators identified in the fluorescence assay were similar to those of the 17 compounds in Fig. 3B.

The 17 compounds were further analyzed for cAMP induction, phosphatase inhibition, dose response, activation mechanism, and reversibility. Fig. 4A summarizes cellular cAMP content after incubation of cells for 10 min with 2 \( \mu \text{M} \) compound in the absence and presence of 0.5 \( \mu \text{M} \) forskolin. Three compounds, CFTR act-07, CFTR act-10, and CFTR act-16, increased cAMP content alone and potentiated the cAMP elevation elicited by the low concentration of forskolin, giving a cAMP content similar to that found for 20 \( \mu \text{M} \) forskolin. The remaining 14 compounds did not increase cellular cAMP when CFTR \( \text{Cl}^- \) current was strongly induced or when given together with 0.5 \( \mu \text{M} \) forskolin. All compounds were also tested for phosphatase inhibition. Incubation of cell homogenates with the phosphorylated peptide for 30 min released 347 ± 19 pmol of phosphate/\( \mu \text{g} \) of protein. None of the compounds (tested at 10 \( \mu \text{M} \)) significantly inhibited phosphatase activity (not shown). The known phosphatase inhibitors, okadaic acid (50 nm), calyculin A (50 \( \mu \text{M} \)), and NaF (50 mm), inhibited phosphate release by 96 ± 2, 95 ± 7, and 83 ± 3\%, respectively. Cell toxicity was measured by the rhodamine uptake assay. None of the 17 compounds at 10 \( \mu \text{M} \) showed significant toxicity when incubated with cell cultures for 24 h.

The fluorescence assay was used to investigate dose response, activation kinetics, and reversibility of CFTR activation. Fig. 4B shows representative dose-response data (I\( _{\text{influx}} \) versus concentration) for two of the CFTR activators that required forskolin (CFTR act-09 and CFTR act-11) and two that did not (CFTR act-05 and CFTR act-16). Fig. 4C, top, shows data for the time course of CFTR activation after the addition of these same compounds. Full activation was found for these and the other strong activators in 2–5 min. Fig. 4C, bottom, shows data for reversibility of CFTR activation in which compounds were incubated with cells for 5 min, and then the fluorescence
Novel High-affinity CFTR Activators

The purpose of this study was to identify and characterize new classes of CFTR activators. We screened collections of diverse, drug-like small molecules that are readily amenable to combinatorial synthesis. Initial screening was performed with individual compounds at 10 μM together with a low concentration of forskolin. The initial screen was designed to broadly identify cAMP-dependent and independent activators of CFTR Cl− conductance that had at least modest cell permeability. The cell lines and screening procedures reported previously (13) were adapted and optimized for automated high-throughput screening to assay >5000 individual compounds per day. In addition, a ΔPhe508-CFTR expressing FRT epithelial cell line was generated that showed low temperature correction of ΔPhe508-CFTR misprocessing. The logistics of compound handling, cell culture, automated screening, and data analysis/data base development for this study presented a considerable challenge. As described, the screening was successful in >90% of 96-well plates as judged by Cl− transport measurements in positive (apigenin dose response) and negative (no added activator) control wells.

The selection of compounds for secondary analysis was based on the magnitude of CFTR activation determined in the primary compound screen. We chose to carry out secondary analysis of 57 compounds that gave greater activation at 10 μM than the reference compound apigenin. After confirming results of the fluorescence assay in CFTR-expressing cells and showing...
no effects on CFTR-null cells, all compounds were screened for stimulation of CFTR Cl$^-$/H$^{+}$ transport by short-circuit current analysis. Seventeen of the compounds gave strong Cl$^-$/H$^{+}$ currents at 1–2 μM concentration and were selected for further analysis. The remaining compounds were substantially less potent and in some cases appeared to be false positives of the fluorescence screening assay. All activators showed low cellular toxicity in the rhodamine uptake cell culture assay. Forskolin-dependence and dose-response measurements indicated that a subset of the strongest CFTR activators required low concentrations of forskolin to activate CFTR.

As mentioned above, the primary modes of acute CFTR activation include increased CFTR phosphorylation by cAMP elevation or phosphatase inhibition and direct interaction with CFTR, as has been shown for genistein and some xanthines (2). Three of the strong CFTR activators produced marked elevation of cell cAMP content, particularly in combination with a low concentration of forskolin. This synergy suggests CFTR activation by phosphodiesterase inhibition. The remaining compounds did not increase cellular cAMP under conditions giving strong CFTR Cl$^-$/H$^{+}$ currents, nor did any of the compounds inhibit cell phosphatase activity, suggesting CFTR activation by a direct interaction mechanism. Single-channel electrophysiology is needed to prove a direct interaction mechanism.

The core structures of the activators shown in Fig. 3B (except for the xanthine CFTR act-01) are unrelated to those of known CFTR activators, which act by altering cellular cAMP concentration or phosphatase activity, or activators that are thought to directly interact with CFTR.
to interact directly with CFTR. Of the 17 strongest activators of CFTR Cl\(^{-}\) conductance, several structurally similar compounds were identified that contain triazine/diazine and thiazolidine-like heterocycles. In addition, many of the other compounds judged by the fluorescence assay to be strong CFTR activators were analogs of some of the 17 compounds. The putative CFTR-interacting activators may be useful to deduce consensus structure(s) for CFTR activation by computational methods and to facilitate CFTR crystallization for structure determination.

The compounds that strongly activated wild-type CFTR were screened for their efficacy in activating G551D-CFTR and ΔPhe\(^{508}\)-CFTR, two mutant CFTRs that cause human CF. Of the 57 strong activators of wild-type CFTR, only three compounds activated G551D-CFTR significantly, indicating that CFTR activation is mutation-specific. Unfortunately, the compounds that activated G551D-CFTR did so weakly (\(K_d\) 10 \(\mu\)M or higher), no better than existing flavonones. However, several of the compounds were effective in activating ΔPhe\(^{508}\)-CFTR. When ΔPhe\(^{508}\)-CFTR-expressing cells were grown at 37°C, where ΔPhe\(^{508}\)-CFTR is retained in the endoplasmic reticulum, none of the compounds activated Cl\(^{-}\) currents when added a few minutes before assay. However several compounds induced strong Cl\(^{-}\) currents with \(K_d\) <1 \(\mu\)M after low temperature correction, which allowed ΔPhe\(^{508}\)-CFTR trafficking to the cell plasma membrane. These acutely acting ΔPhe\(^{508}\)-CFTR activators may be useful to maximize ΔPhe\(^{508}\)-CFTR Cl\(^{-}\) transport in combination with compounds that correct ΔPhe\(^{508}\)-CFTR misprocessing, for example in improving the sensitivity of a screen to identify compounds that correct ΔPhe\(^{508}\)-CFTR misprocessing.

In summary, the screening of a collection of 60,000 diverse drug-like compounds revealed new chemical classes of CFTR activators with activation potencies down to 200 nM. The activators were CFTR-specific, rapid, reversible, and nontoxic. Most of the compounds activated CFTR Cl\(^{-}\) currents without increasing cellular cAMP content or inhibiting cellular phosphatase activity, suggesting direct CFTR interaction. Some of the compounds activated CFTR only in the presence of mild cAMP activation. Also, several of the compounds strongly activated the CF-causing CFTR mutant ΔPhe\(^{508}\) at submicromolar concentrations after correction of mistrafficking by growth at low temperature. The compounds identified here may be useful in elucidating CFTR activating mechanisms and as leads in CF drug development.

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