Novel CFTR Chloride Channel Activators Identified by Screening of Combinatorial Libraries Based on Flavone and Benzoquinolizinium Lead Compounds

The flavonoid genistein and the benzo[c]quinolizinium MPB-07 have been shown to activate the cystic fibrosis transmembrane conductance regulator (CFTR), the protein that is defective in cystic fibrosis. Lead-based combinatorial and parallel synthesis yielded 223 flavonoid, quinolizinium, and related heterocyclic compounds. The compounds were screened for their ability to activate CFTR at 50 μM concentration by measurement of the kinetics of iodide influx in Fisher rat thyroid cells expressing wild-type or G551D CFTR together with the green fluorescent protein-based halide indicator YFP-H148Q. Duplicate screenings revealed that 204 compounds did not significantly affect CFTR function. Compounds of the 7,8-benzoflavone class, which are structurally intermediate between flavones and benzoc[quinoliziniums], were effective CFTR activators with the most potent being 2-(4-pyridinium)benzo[h]1H-chromen-4-one bisulfate (UCc15-029). Compounds of the novel structural class of fused pyrazolo heterocycles were also strong CFTR activators with the most potent being 3-(3-butylnyl)-5-methoxy-1-phenylpyrazole-4-carboxaldehyde (UCc15-180). A CFTR inhibitor was also identified. The active compounds did not induce iodide influx in null cells deficient in CFTR. Short-circuit current measurements showed that the CFTR activators identified by screening induced strong anion currents in the transfected cell monolayers grown on porous supports. Compared with genistein, the most active compounds had up to 10 times greater potency in activating wild-type and/or G551D-CFTR. The activators had low cellular toxicity and did not elevate cellular cAMP concentration or inhibit phosphatase activity, suggesting that CFTR activation may involve a direct interaction. These results establish an efficient screening procedure to identify CFTR activators and inhibitors and have identified 7,8-benzoflavones and pyrazolo derivatives as novel classes of CFTR activators.

The most common lethal genetic disease, cystic fibrosis (CF), is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein CFTR (1). CFTR is a cAMP-regulated epithelial cell membrane Cl⁻ channel that seems also to regulate the activities of other membrane proteins (2). Normally Cl⁻-permeable epithelial cells in airways, pancreas, and other tissues become Cl⁻-impermeable in CF, resulting in defective salt, water, and protein transport. Although the exact mechanism by which decreased CFTR Cl⁻ permeability produces lung and pancreatic disease in CF remains unclear, it is generally believed that restoration of CFTR Cl⁻ permeability will be clinically beneficial. An important goal in CF research is thus the identification of small molecule activators of CFTR.

Activators of CFTR chloride permeability can function by a number of direct and indirect mechanisms including increased cAMP production, inhibition of phosphodiesterase or phosphatase activities, or direct interactions with CFTR. Several chemical classes of activators have been identified including flavones/isoflavones (e.g. genistein, Refs. 3 and 4), benzo[c]quinoliziniums (MPB-07, Ref. 5), xanthines (isobutylmethylxanthine and 8-cyclopentyl-1,3-dipropylxanthine, Refs. 6–8), and benzimidazoles (NS004, Ref. 9). Flavones/isoflavones such as genistein and apigenin are thought to interact directly with the nucleotide binding fold regions of CFTR and not by the inhibition of tyrosine kinases (10, 11), although the exact mechanism has not been determined (12). There has been considerable interest in developing improved flavone-type CFTR activators because of their consistent and strong activation of wild-type and mutant CFTR in different cell types (13–15). The benzo[c]quinolizinium MBP-07 also seems to activate CFTR without elevating intracellular cAMP or ATP concentrations or affecting the activities of several known protein phosphatases (5).

The purpose of this study was to apply a high throughput screening assay for the discovery of novel CFTR activators. Preliminary combinatorial libraries were synthesized based on the flavone and benzo[c]quinolizinium structures. We chose these lead compounds because they are the most likely of the known activators to interact directly with CFTR, and they share a common structural motif amenable to the design of hybrid structures. In addition, as reported separately (4, 16–21), we have developed solution and solid-phase synthesis and purification methods for the efficient large scale production of test compounds. We report here the screening of purified test...
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compounds for CFTR-activating potency by a cell-based halide transport assay that utilized fluorescent epithelial cells stably expressing wild-type or G551D CFTR together with the green fluorescent protein halide indicator YFP-H148Q. YFP-H148Q fluorescence is decreased by halides by a rapid shift in \( pK_a \) upon halide binding to a site near its tri-amino acid chromophore (22). The screen revealed new classes of CFTR activators and a CFTR inhibitor, which were characterized further in terms of potency, CFTR specificity, activation mechanism, and the ability to induce transepithelial chloride currents in polarized epithelial cells.

EXPERIMENTAL PROCEDURES

Generation of Combinatorial Compound Library—(See Supplemental Material for a complete list of compounds and their structures.) Unless otherwise indicated, the flavonoids screened in the present study were prepared using the recently reported modification (23) of the conventional Baker-Venkatakrarman flavone synthesis (24, 25). The flavones UCCr-016–UCCr-021 were synthesized using a different modification of the Baker-Venkatakrarman flavone synthesis (26). All 2-aryl-4-quinolones and 2-aryl-quinoline-4-thiones were prepared using our previously described protocol (19). The flavone UCCr-054 was prepared as described (4), and the flavones UCCr-027, UCCr-028, and UCCr-034–UCCr-038 were prepared from 5-hydroxy-4-iodoflavone via Suzuki coupling (27) using the following representative experimental procedure: 3-thiopheneboronic acid (1.5 eq, 15 mg), Pd(PPh₃)₄ (2.5 mol %, 1.7 mg, 0.00157 mmol), and K₂CO₃ (2.7 eq, 2.3 mmol, 2.54 mmol) were added to a stirred solution of the 5-hydroxy-4-iodoflavone (1.0 eq, 25 mg, 0.0628 mmol) in a mixture of dimethoxyethane (0.75 ml) and water (0.1 ml). The mixture was refluxed at 85 °C for 9 h. After cooling, the solvent was removed by rotary evaporation. The solid residue was dissolved in dichloromethane and extracted twice with 30 ml of water. The combined organic layers were washed with brine and dried using anhydrous sodium sulfate. After concentration, the solid residue was redissolved with a minimum quantity of hot ethyl acetate followed by the slow addition of hexane to allow crystallization. The solids were then filtered and washed with excess 1:1 hexane/ethyl acetate to obtain 10.5 mg of UCCr-037 (52% yield) as a light cream-colored solid. Compound identity and purity were confirmed by \( ^1H \) and \( ^{13}C \) NMR and thin layer chromatography. Quinolinizinium salts were synthesized using the procedure described by Beeg et al. (5). The (ω-hydroxy)alkylpyridinium and aza-cyanine analogs were prepared using our previously published procedures (16, 17). The fused pyrazolo heterocycles were synthesized as reported (18). Tetrazines and isoxazole heterocycles were prepared as described (20, 21).

The core structures of indicated compound classes are shown. See Supplemental Material for the structures of all compounds synthesized and screened.

Cell Culture and Transfection—Fischer rat thyroid (FRT) cells expressing the human wild-type CFTR or CFTR-G551D were transfected with the plasmid pCDNA3.1 (Invitrogen) containing the cDNA encoding YFP-H148Q and selected in G418 (0.75 mg/ml). Clonal populations were obtained by repeated limited dilution and cloning rings. Cells were cultured in Coon’s modified F-12 medium supplemented with 5% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Halide Transport Assay—Cells were plated in 96-well black microplates (Corning Costar) at a density of 20,000 cells/well using a Labsystems multidirop apparatus. After 24–48 h, the cells were washed three times with phosphate-buffered saline using a Labsystems cell wash apparatus and were incubated at 37 °C for 30 min. The residual volume of phosphate-buffered saline was 40 μl/well. The assay was performed in a PLUOstar Galaxy microplate reader (BMG LabTechnologies, Inc.) equipped with pH500/20X (50 ± 10 nm) excitation and pH535/30M (535 ± 15 nm) emission filters (Chroma Technology Corp.) and syringe pumps. YFP fluorescence in each well was monitored continuously for 100 s, and data were binned in 0.5-s intervals. At 5 s after the start of fluorescence recording, a syringe pump delivered 110 μl of a modified phosphate-buffered saline in which 137 mM NaCl replaced NaCl to give an extracellular [1] of 100 mM. After an additional 15 s, a second syringe pump delivered 50 μl of a 100 mM I−-containing solution including specified concentrations of forskolin. The assays were performed at 37 °C.

cAMP activity was measured using the BIOTRAK enzymatic immunoassay (Amersham Pharmacia Biotech). FRT cells expressing CFTR were cultured in 96-well plates. After washing and incubation in phosphate-buffered saline, the cells were incubated for 10 min with specified concentrations of forskolin, UCCr-029, or UCCr-180 and then lysed with the reagent provided by the kit. The lysates were assayed for cAMP content in triplicate according to manufacturer’s instructions. Phosphatase activity was determined with a nonradioactive assay kit (ProMega). The reaction was terminated by the addition of the dye/additive mix-
written using LabView 6I software (National Instruments). At the beginning of the experiment, the basolateral membrane was permeabilized by adding 250 μg/ml amphotericin B. Permeabilization (developing over ~30 min) was monitored by changes in transepithelial resistance. Forskolin was added at indicated concentrations in both hemichambers, and test compounds were added on the apical side only. In these measurements the basolateral membrane was permeabilized with amphotericin B, and a Cl\(^-\) gradient was established to measure CFTR-mediated Cl\(^-\) transport directly as reported (29, 30). This maneuver eliminates possible contributions of basolateral membrane channels. A Cl\(^-\) gradient was established to generate a driving force for basolateral to apical Cl\(^-\) transport at zero transepithelial potential difference.

**RESULTS**

Fig. 1 shows core structures of the classes of compounds that were synthesized. The compounds are referred to as UCCF-01 (University of California-cystic fibrosis) through UCCF-223 and have been grouped in the following structural classes: flavonoids, quinoliniziniums, pyridiniums, azacyanines, isoxazoles, and fused pyrazole heterocycles. Fig. 1 (bottom row) shows the structures of the reference compound genistein and two CFTR activators that were investigated in detail.

Each compound was screened individually for its potency as a CFTR activator/inhibitor. Fig. 2A shows the fluorescence assay. FRT cells coexpressing human wild-type CFTR or G551D CFTR (which causes CF) and YFP-H148Q were cultured on 96-well plates for the monitoring of YFP fluorescence in a plate reader. The FRT cells and assay conditions were chosen to minimize CFTR-independent halide transport and basal (prior to cAMP simulation) CFTR halide transport. After recording baseline fluorescence, an osmotically matched I\(^-\)-containing solution was added to the Cl\(^-\)-containing solution bathing the cells to establish a 100 mM inwardly directed I\(^-\) gradient (Fig. 2A, left). There was little I\(^-\) influx (decrease in fluorescence) prior to the activation of wild-type CFTR under the experimental conditions used here. Subsequent activation by forskolin resulted in dose-dependent I\(^-\) influx and decreased cell fluorescence. Neither activation of G551D CFTR nor of null cells that do not express CFTR was observed under these conditions (data not shown). As depicted schematically (Fig. 2A, middle and right panels), a test compound could (a) have no effect, (b) activate CFTR directly (prior to forskolin addition), (c) activate CFTR in synergy with forskolin (after forskolin addition), or (d) inhibit CFTR.

In the initial screen, test compounds were added to the cell
bathing solution at 50 μM concentration just prior to the assay. For screening of the cells expressing wild-type CFTR, a submaximal concentration of forskolin (250 nM) was added during the assay to probe for synergy with the test compounds with cAMP activation. Fig. 2B summarizes the data from duplicate screening of the compound library. The ordinate is the maximum normalized slope of the fluorescence time course, representing I influx (in mV/s) measured after I addition. Although most of the compounds were inactive, several compounds activated CFTR strongly in the absence of forskolin, as seen by the prompt fluorescence decrease upon I addition. Fig. 2B (inset) provides representative original curves from the screen showing activation of wild-type CFTR by the compounds UCCF-027, UCCF-029, UCCF-031, and UCCF-180. The novel activators included compounds of the novel 7,8-diphenylamine-2-carboxylate. The effect of forskolin is shown in the same compounds that activated wild-type CFTR also activated G551D CFTR, although relative activating potencies differed, and a few compounds activated preferentially wild-type CFTR (e.g. UCCF-180) or G551D CFTR (e.g. UCCF-023 and UCCF-030). Representative original fluorescence curves are shown in Fig. 2C (inset).

Further analysis was done on compounds with apparent CFTR-activating potency. Except for compound UCCF-152, repeat assays of the putative CFTR activators confirmed the results of the original screens. Additional screening using a different cell line (Chinese hamster ovary cells expressing CFTR and YFP-H148Q) confirmed the results in FRT cells (data not shown). None of the putative CFTR activators induced halide flux in cells not expressing CFTR. Preliminary dose-response data (concentration range 1–50 μM) were generated to identify those compounds warranting further investigation. Fig. 3A depicts a dose-dependence for the activation of wild-type CFTR by UCCF-029 and UCCF-180, showing substantially better potency than the reference compound genistein. Fig. 3B shows a similar comparison for the activation of G551D CFTR. Genistein was active at >50 μM, whereas UCCF-023, UCCF-027, UCCF-028, and UCCF-030 induced significant iodide influx at 6.25–12.5 μM.

Electrophysiological measurements of Cl current were done to confirm CFTR activation and to compare activation potencies. Short-circuit current was measured in polarized monolayers of FRT cells expressing wild-type or G551D CFTR. Fig. 4A shows short-circuit current in response to the activation of wild-type CFTR by genistein versus UCCF-029 and UCCF-180. The induced Cl current was inhibited by the CFTR blocker diphenylamine-2-carboxylate. The effect of forskolin is shown for comparison. UCCF-029, the most potent activator of wild-type CFTR in the initial screening, induced a strong Cl current at 5–10 μM with a significant effect at 1–2 μM, whereas genistein was not effective at the same concentrations. At 5 and 10 μM concentrations, UCCF-029 was 10 and 16 times more effective than genistein in increasing short-circuit current (in μA/cm², n = 5; S.E. = 61 ± 16 versus 5.7 ± 3 [5 μM]; 115 ± 28 versus 7 ± 2 [10 μM]). As found in the plate-reader assay, UCCF-180 induced significant short-circuit current at concentrations lower than those required for genistein. Similar experiments showed no increase in short-circuit current in nontransfected FRT cells, supporting the conclusion that these compounds activate CFTR. The CFTR inhibitor UCCF-019 identified in the screening was confirmed by short-circuit current analysis (data not shown). We found that compounds of the benzozquinolizinium class including the lead compound MPB-07 (freshly synthesized and dissolved) were not effective in activating CFTR in the plate-reader assay or by short-circuit current analysis. It is unclear whether cell-type differences or...
other factors account for the absence of MPB-07 activation of CFTR in our experiments.

Fig. 4B shows short-circuit current analysis of G551D CFTR activation by genistein versus UC CF-030. UC CF-030 was remarkably more potent than genistein, which is in agreement with the fluorescence plate-reader data. Interestingly, the activator of wild-type CFTR, UC CF-180, was ineffective in activating G551D CFTR. The compounds UC CF-023, UC CF-027, and UC CF-028 were also effective in inducing short-circuit current in cells expressing G551D CFTR (data not shown).

All compounds were screened for cell toxicity using a dihydrorhodamine accumulation assay. At 50 μM concentrations for 24 h, the reference compound genistein caused ~20% growth inhibition. All compounds with activating potency on wild-type and/or G551D CFTR were nontoxic (cell growth >90% of control) except for UC CF-027, which was similar to genistein. To investigate the CFTR-activating mechanism, the CFTR activators were tested for their ability to elevate intracellular cAMP concentration and inhibit cell phosphatase activity. Using an enzymatic immunoassay, no significant elevation in cAMP concentration was found for 10-min incubations with the activators UC CF-029 at 5 μM and UC CF-180 at 25 μM (Fig. 5A), concentrations that induced short-circuit currents of 61 ± 16 and 53 ± 12 μA/cm², respectively. These values were significantly higher than that induced by 1 μM forskolin (19 ± 4 μA/cm²), which produced a substantial elevation in intracellular cAMP concentration. Fig. 5B summarizes the results of an assay of cell phosphatase activity, showing strong inhibition by okadaic acid and NaF. Total cell phosphatase activity was not significantly inhibited at concentrations of UC CF-029 and UC CF-180 that markedly stimulated CFTR. Together the data suggest that the CFTR-activation mechanism may involve direct compound-CFTR interactions.

**DISCUSSION**

This study was designed to identify new CFTR activators that interact directly with CFTR. Structure-based drug design is not yet possible for CFTR because of the very limited knowledge about CFTR tertiary structure. We chose to generate a combinatorial compound library based on two lead compounds, flavones and benzo[c]quinoliziniums, which are believed to activate CFTR Cl⁻ conductance by direct interaction with the CFTR molecule. A secondary purpose of this study was to establish the utility of a new cell-based screening assay to efficiently quantify the activity of putative CFTR activators and inhibitors. A number of technical challenges were encountered including the synthetic organic chemistry, cell line development, and transport assay optimization.

Several novel CFTR activators were identified as well as a CFTR inhibitor. Interestingly, the structures of a potent class of compounds, the 7,8-benzo[quinoliziniums. Activators of the novel class of fused pyrazole...
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heterocycles were also identified. Although these compounds (such as UCcr-180) were less potent than the most potent benzoflavonones, they represent a new class of CFTR activators. The CFTR activators did not induce Cl⁻ currents in null cells, and short-circuit current analysis showed that they had activating potencies for wild-type and G551D CFTR that were substantially better than the existing lead compounds. The CFTR activators were nontoxic in the cell culture model and activated CFTR without measurable elevation in intracellular cAMP concentration or inhibition of total cell phosphatase activity. Single channel measurements will be required to establish whether these compounds interact directly with CFTR.

Interestingly, the order of activating potencies of the CFTR activators on wild-type versus G551D CFTR was different. For example, UCcr-029 was the most potent compound in activating wild-type CFTR but had less effect on G551D CFTR. In contrast, UCcf-023, UCcf-028, and UCcf-030, which were substantially less potent than UCcf-029 in activating wild-type CFTR, were very potent in activating G551D CFTR in synergy with forskolin. As additional structural information on CFTR becomes available, the compounds with differential potencies discovered here may be useful in identifying critical structural motifs important for CFTR activation. For the most common CFTR mutant causing cystic fibrosis in humans, ΔF508 CFTR, the activators identified here may be useful in synergy with compounds that correct the intracellular processing defect of this mutant.

Although our main aim was to identify CFTR activators, the assay permits the identification of CFTR inhibitors. It was found that the flavone UCcf-019 significantly reduced CFTR-mediated halide transport. Further studies are needed to determine whether the reduced halide transport involves direct CFTR binding or inhibition of the CFTR-activating pathway. CFTR inhibitors are important in basic cystic fibrosis research and may be useful clinically in the treatment of secretory diarrheas such as cholera.

The fluorescence assay reported here should be useful in high throughput screening of other lead-based compound libraries as well as random combinatorial libraries. The assay permits the rapid quantitative measurement of cAMP-independent and -dependent halide permeability using a commercial automated fluorescence plate reader. The goal in identifying CFTR activators is to discover clinically useful drugs that will activate mutant CFTR molecules in cystic fibrosis. The underlying assumption in CF drug discovery is that activation of CFTR in cell culture models is a good surrogate marker for in vivo efficacy in improving clinical outcome in cystic fibrosis patients. Although this assumption is supported by a considerable body of data on the biology and function of CFTR, rigorous validation will require animal and human testing of potent nontoxic CFTR activators.

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