Identification of New Small Molecule Inhibitors of Cystic Fibrosis Transmembrane Conductance Regulator Protein: In Vitro and in Vivo Studies

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Cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cAMP-regulated chloride channel that has been proposed as a pharmacological target to reduce intestinal fluid loss in cholera. The aim of this study was to identify new CFTR inhibitors by high-throughput screening. Screening of 50,000 drug-like small molecules was performed using a cell-based assay of iodide influx in Fisher rat thyroid (FRT) cells co-expressing human CFTR and halide-sensitive yellow fluorescent protein (YFP-H148Q). Two new CFTR inhibitors, 2-[N-(3-hydroxy-4-carboxyphenyl) amino]-4-(4-methylphenyl)-thiazole (INH 1) and 1-acetyl-5-bromo-2,3-dihydro-1-(2,3,4-tetrahydro-1-naphthalenyl)-1H-Indole-7-sulfonamide (INH 2), were identified. They were then determined for potency, reversibility and specificity by electrophysiological methods, and for in vivo efficacy in mouse model of cholera toxin-induced intestinal fluid secretion. INH 1 and INH 2 reversibly inhibited cAMP-activated apical chloride current in FRT cells with $K_i$ of 15 and 20 $\mu M$, respectively. Similarly, in short-circuit current analysis in human colonic epithelial cell lines (T84 cells), cAMP-activated chloride secretion was inhibited by INH 1 and INH 2 with $K_i$ of 24.5 and 25.3 $\mu M$, respectively. Calcium-activated chloride secretion in the T84 cells was markedly inhibited by 100 $\mu M$ of INH 1, but was unaffected by 100 $\mu M$ of INH 2. In vivo studies in mice showed that a single intraperitoneal injection of INH 1 (3 mg/kg) reduced cholera toxin-induced intestinal fluid secretion by 40%, whereas INH 2 produced no effect. Our results indicate that INH 1 could be a new class candidate for a blocker of cholera toxin-induced intestinal fluid secretion as well as a CFTR inhibitor.

Key word diarrhea; cystic fibrosis; chloride channel; drug discovery

Excessive intestinal fluid secretion plays an important role in the pathogenesis of secretory diarrhea. It is a passive process driven by the active secretion of ion, predominantly chloride.1,2) The cellular transport mechanism of intestinal chloride secretion is well defined.1) Chloride is taken up across the basolateral membrane via a Na$^+$/K$^+$/2Cl$^-$.cotransporter (NKCC1) and exits across the apical membrane via a cAMP-sensitive (CFTR) and calcium-sensitive (CaCC) chloride channels. Basolateral potassium channels support chloride secretion by allowing for potassium recycling, whereas the energy for the process is supplied by the activity of a basolateral Na$^+$/K$^+$.ATPase. An increase in chloride secretion is usually mediated by elevated intracellular levels of second messengers, such as cAMP and calcium. Secretory responses to these two second messengers are distinct in that responses to cAMP agonists are sustained, whereas those to Ca$^{2+}$-mediated agonists are transient. cAMP was found to be a primary second messenger involved in the pathogenesis of diarrhea in cholera, whereas calcium was thought to mediate enhanced intestinal chloride secretion in diarrhea caused by rotavirus infection and diarrhea that is associated with the use of aspartyl protease inhibitors,2) an antiviral drug for human immunodeficiency virus-infected patients.

Cholera is life-threatening secretory diarrhea resulting from intestinal infection with gram negative bacteria Vibrio cholera.3) Cholera toxin, an enterotoxin produced by Vibrio cholera, acts on enterocytes to increase intracellular cAMP, which in turn leads to massive CFTR-dependent intestinal chloride and, secondarily, fluid secretion.1) The volume of secreted fluid far exceeds the intestinal absorptive capacity, resulting in severe intestinal fluid loss and associated morbidity and mortality in affected individuals.1,2) Therefore, there are considerable efforts focused on the developing of antisecretory agents for patients suffering from cholera.3) On the basis of both in vitro5) and in vivo5) studies, one of the proposed targets to relief the symptom is an inhibition of CFTR, a cAMP-activated chloride channel that provides the apical route for intestinal chloride secretion.1,2)

At present, two classes of potent CFTR inhibitors (thiazolidinone and glycine hydrazide) have been identified by high-throughput screening.5,7) Both are effective in reducing cholera toxin-induced intestinal fluid secretion in mice.5,7) The lead thiazolidinone compound, CFTR$_{inh}$-172, reversibly inhibited CFTR with $K_i$ of 0.3 $\mu M$, presumably by binding to the CFTR's nucleotide binding domain 1.8,9) It has no effect on other chloride channels and multidrug resistance transporter proteins, but also has poor water solubility ($\sim 20$ $\mu M$).5,9) In contrast, the lead glycine hydrazide compound, GlyH-101, has much greater water solubility ($\sim 1$ mm) and CFTR inhibition with $K_i$ of 5 $\mu M$.7) However, its inhibitory effect on calcium-activated chloride channels was also observed at a concentration (50 $\mu M$) that completely inhibits CFTR activity.7) Recently, highly polar, water soluble and cell-impermeable glycine hydrazide-based CFTR inhibitors were synthesized and shown to inhibit CFTR activity with $K_i$ of 2—5 $\mu M$.10)

In spite of the above mentioned favorable properties of current lead compounds, efforts to develop them as antidiarrheal drugs may be hampered by such drawbacks as poor water solubility and potential off-target effects. Therefore, this study was aimed at identifying new classes of CFTR inhibitors with equal or better properties as additional compounds of choice. We performed high-throughput screening of 50,000 chemically diverse drug-like small molecules and identified two new classes of CFTR inhibitors. They were tested for their inhibitory potency, reversibility and speci-
ficiency by electrophysiological measurements in Fisher rat thyroid cells (FRT cells) and T84 cells and for their in vivo effic‌acy in a mouse closed loop model of cholera toxin-induced intestinal fluid secretion.

MATERIALS AND METHODS

Chemicals A library of 50,000 small molecules (molecular weight ~250—550 Dalton) was selected from ChemDiv (San Diego, CA, U.S.A.) using algorithms designed to maximize chemical diversity and drug-like properties. They were stored frozen as 2.5 mM stock solutions in DMSO. CFTRinh-172 was a gift from Professor A.S. Verkman, University of California, San Francisco (San Francisco, CA, U.S.A.). Cholera toxin was obtained from List Biological Laboratories (Campbell, CA, U.S.A.). Forskolin, apigenin, 3-isobutyl-1-methylxanthine (IBMX), amiloride, amphotericin B, carbamol and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

High-Throughput Screening and Cell-Based Iodide Influx Assays Screening was performed using an integrated system (Beckman Coulter, Fullerton, CA, U.S.A.) consisting of a 3-meter robotic arm, CO2 incubator, plate washer, liquid handling work station, barcode reader, delidding station, and a liquid transport system (Beckman Coulter, Fullerton, CA, U.S.A.) consisting of a 3-meter robotic arm, CO2 incubator, plate washer, liquid handling work station, barcode reader, delidding station, and two fluorescence plate readers (Optima; BMG Lab Technologies, Offenburg, Germany), each equipped with two syringe pumps and HQ500/2X (500 · cm2) excitation and HQ535/30M (535 ± 15 nm) emission filters (Chroma Technology Corp., Brattleboro, VT, U.S.A.). Fisher rat thyroid (FRT) cells stably expressing wild-type human CFTR (hCFTR) and YFP-H148Q, a mutant yellow fluorescent protein sensitive to halide, were cultured on 96-well black-wall plates at density of 20,000 cells/well 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. Cells were grown in a humidified CO2 incubator for 24 h before the screening assays. For screening, FRT cells in 96-well plates were washed three times, and CFTR halide conductance was then activated by incubation for 15 min with an activating cocktail containing 10 μM forskolin, 20 μM apigenin, and 100 μM IBMX. Test compounds (25 μM final concentrations) were added 5 min before an assay of iodide influx in which cells were exposed to a 100 mM inwardly directed iodide gradient. YFP fluorescence was recorded for 2 s before and 12 s after creation of the iodide gradient. Initial rates of iodide influx were computed from the time course of fluorescence decrease following establishment of iodide gradient.

Apical Cl⁻ Current and Short-Circuit Current Measurements FRT and T84 cells were cultured on Snapwell filters with 1 cm² surface area (Corning-Costar, Acton, MA, U.S.A.) to resistances of >1000 Ω · cm² as described previously. Filters were mounted in customized chambers. For apical Cl⁻ current measurements in FRT cells, the basolateral hemichamber was filled with buffer containing 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 1 mM CaCl2, 0.5 mM MgCl2, 10 mM Na-HEPES (pH 7.3) and 10 mM glucose. The basolateral membrane was permeabilized with amphotericin B (250 μg/ml) for 30 min before measurements. In the apical solution, 65 mM NaCl was replaced with 65 mM sodium gluconate, and concentration of CaCl2 was increased to 2 mM. For short-circuit current measurements in (nonpermeabilized) T84 cells, both hemichambers contained Kreb’s solution containing 120 mM NaCl, 25 mM NaHCO3, 3.3 mM KH2PO4, 0.8 mM K2HPO4, 1.2 mM MgCl2, 1.2 mM CaCl2 (pH 7.3) and 10 mM glucose. Solutions were bubbled with 95% O2/5% CO2 and maintained at 37 °C. Apical Cl⁻/short-circuit current was recorded using a DVC-1000 voltage-clamp (World Precision Instruments, Sarasota, FL, U.S.A.) with Ag/AgCl electrode and 1 mKCl agar bridge.

Intestinal Fluid Secretion Measurements Mice (ICR strain, 35—45 g, National Laboratory Animal Center, Bangkok, Thailand) were deprived of food for 24 h and anesthetized with intraperitoneal injection of ketamine (40 mg/kg) and xylazine (8 mg/kg). Body temperature was maintained at 36—38 °C using a heating pad. Following a small abdominal incision, three closed mid-jejunal loops (length 2—3 cm) were isolated by sutures. Loops were injected with 100 μl of phosphate-buffered saline (PBS) or PBS containing cholera toxin (1 μg). In some experiments, the test compounds were prepared in 100 μl DMSO and injected intraperitoneally before abdominal closure. The abdominal incision was closed with suture and mice were allowed to recover from anesthesia. After 4 h, the mice were anesthetized, intestinal loops were removed, and loop length and weight were measured to quantitate net fluid secretion. Mice were then killed by an overdose of ketamine and xylazine. All animal protocols were conducted in accordance with principles and guidelines of the Laboratory Animal Ethical Committee of Mahidol University, Thailand.

RESULTS

Identification of CFTR Inhibitors by High-Throughput Screening To identify new CFTR inhibitors, we performed high-throughput screening of 50,000 drug-like small molecules using the iodide influx assay in FRT cells stably expressing human wild type CFTR and halide-sensitive yellow fluorescence protein H148Q (YFP-H148Q). Co-expression of the YFP-H148Q provided a quantitative fluorescence read-out of inhibition potency of test compounds. The screening procedure is shown in Fig. 1A. All compounds (25 μM) were tested for their CFTR inhibitory effects following CFTR activation by cocktail containing 10 μM forskolin (an adenyly cyclase activator), 100 μM IBMX (a phosphodiesterase inhibitor) and 20 μM apigenin (a flavone-type direct CFTR activator). The rationale for CFTR activation by multiple mechanisms was to identify inhibitors that blocked the CFTR Cl⁻-transporting pathway directly rather than blocking a more proximal step(s) in the activation pathway. In this assay, CFTR channel activity was determined from the rate of decrease in YFP fluorescence after addition of iodide-containing solution. Representative fluorescence recordings are shown in Fig. 1B, including those from a negative control well (PBS alone), a positive control well (activating cocktail plus 1% DMSO) and wells containing activating cocktail plus active test compounds (INH 1 and INH 2). CFTRinh-172 (5 μM), a potent and specific CFTR inhibitor, produced a significant decrease in the negative slope. INH 1 and INH 2 also reduced iodide influx by >50% and were subsequently found to be 2-[N-(3-hydroxy-4-carboxyphenyl)amino]-4-(4-methylphenyl)-thiazole (INH 1) and 1-acetyl-5-bromo-2,3-
dihydro-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1H-indole-7-sulfonamide (INH 2), respectively. Their chemical structures are shown in Fig. 1C.

**Dose–Response Relationship and Reversibility of the CFTR Inhibitors in FRT Cells** To determine potency of CFTR inhibitory actions of INH 1 and INH 2, dose–response relationship was determined by iodide influx assays and apical chloride current measurements in the FRT cells. Figure 2A shows representative fluorescence recordings from dose–response studies of INH 1 (left) and INH 2 (right). Both compounds inhibited iodide influx in a dose-dependent manner. The apical chloride current measurement was performed using basolaterally permeabilized FRT cells with an apically directed chloride gradient. After basolateral permeabilization by amphotericin B (250 µg/ml), the basolateral membrane is electrically eliminated, so that the changes in currents induced by drugs should reflect the changes in conductance of the apical membrane, where CFTR is primarily located. In order to measure CFTR-mediated ion currents across this membrane, a driving force, chemical chloride gradient, was introduced. Representative current recordings from dose–response studies of INH 1 and INH 2 are shown in Fig. 2B. Forskolin treatment (10 µM) elicited CFTR-mediated apical chloride current that was inhibited by each compound in a dose-dependent fashion. Based on the observation that INH 2 precipitated at 100 µM, thus, dose–response analysis of INH 2 was performed using data from doses of 1 to 50 µM. A summary of the data is shown in Fig. 2C, with the calculated Kᵦ of INH 1 and INH 2 being 15 and 20 µM, respectively.

Similar dose–response relationships were obtained in experiments using 50 µM dibutyryl-cAMP (a cell-permeable cAMP) or 50 µM genistein (a direct CFTR activator) as a CFTR agonist (data not shown).

The reversibility of CFTR inhibition by each compound was tested in apical chloride current measurements. As shown in Fig. 3, the inhibition of chloride current was reversed after removal of INH 1 and INH 2 from the bathing medium.
solutions. As a control, more than 80% of the recovered chloride current was inhibited by the specific CFTR inhibitor CFTRinh-172 (20 μM).

Dose–Response Studies of CFTR Inhibitors in Short-Circuit Current Measurement in T84 Cells

T84 cells are human cancer colonic cells that have crypt-like properties and are widely used to study intestinal chloride secretion. To test the potency of the CFTR inhibitors in human intestinal cells, dose–response experiments of each compound were conducted in short-circuit current analysis using non-permeabilized T84 cells (with no chloride gradient). In the presence of amiloride (10 μM) to block epithelial sodium channel (ENaC), INH 1 (Fig. 4A) and INH 2 (Fig. 4B) inhibited cAMP-activated short-circuit current in a dose-dependent manner. Data from 3—5 experiments are summarized in Fig. 4C. Kₘ of INH 1 and INH 2 were 24.5 and 25.3 μM, respectively, with more than 90% inhibition at higher doses.

Effect of the New CFTR Inhibitors on Calcium-Activated Chloride Secretion in T84 Cells

Another physiologically important component of intestinal chloride secretion, in addition to a cAMP-dependent process, is a calcium-dependent pathway. We assessed the specificity of the CFTR inhibitors as blockers of Ca²⁺-activated chloride secretion using short-circuit current measurements in nonpermeabilized T84 cells. Amiloride (10 μM) and CFTRinh-172 (20 μM) were present in the apical solution for entire experimental periods to prevent contribution of ENaC and CFTR to short-circuit current measured. An acetylcholine agonist, carbachol, was added into a basolateral solution to stimulate Ca²⁺/H⁺-activated chloride secretion by a mechanism involving elevation of intracellular calcium. Carbachol-stimulated chloride current, as deduced from the peak values, were unaffected by 100 μM of INH 2, but completely abolished by 100 μM of INH 1 (Fig. 5A). Figure 5B shows a summary of the data. Peak carbachol-activated short-circuit currents in cells treated with INH 2 (2.23±0.47 μA/cm²) were not significantly different from that of control experiments (2.16±0.29 μA/cm²). In contrast, INH 1 dramatically inhibited carbachol-activated chloride current, with the peak carbachol-activated short-circuit current being 0.05±0.15 μA/cm².

In Vivo Efficacy of CFTR Inhibitors in a Closed-Loop Model of Cholera Toxin-Induced Intestinal Fluid Secretion

In vivo efficacy testing of new CFTR inhibitors was performed in a closed-loop mouse model of cholera toxin-induced intestinal fluid secretion. Mid-jejunal loops injected with phosphate-buffered saline (PBS) with or without cholera toxin (1 μg/loop) were produced, and intestinal fluid secretion induced by cholera toxin over 4 h was quantified from loop weight-to-length ratio. A single intraperitoneal injection of INH 1 (3 mg/kg) at the time of cholera toxin injection significantly reduced cholera toxin-induced fluid secretion by 40% (from 0.195±0.003 g/cm to 0.133±0.005 g/cm) (Fig. 6). In contrast, an intraperitoneal injection of INH 2 (3 mg/kg) had no antisecretory effect and associated with precipitation of the compounds in mouse abdomen (data not shown).

DISCUSSION

The purpose of this study was to identify new small molecule CFTR inhibitors by high-throughput screening and to characterize their properties. Two new small molecule CFTR inhibitors, INH 1 and INH 2, were identified from screening a library of 50,000 compounds and shown to inhibit CFTR-mediated chloride current in CFTR-transfected FRT cells and human crypt-like epithelial cells (T84 cells). CFTR inhibi-
chloride and secondarily fluid secretion into the intestinal lumen. In addition, Na+ and fluid absorption are inhibited in this condition, resulting in massive intestinal fluid loss and death if untreated. Currently, ORS remains the mainstay therapy for cholera, it could reduce the mortality rate from about 50% to only 1%. However, it only corrects the loss of fluid and electrolytes but has no effect on reducing voluminous fluid secretion, severity and duration of diarrhea. Cell culture and in vivo studies have indicated CFTR as a principal apical route for chloride secretion induced by cholera toxin. Inhibition of CFTR has therefore been proposed as a potential pharmacological target of intervention and to reduce severity and duration of diarrhea in patients suffering from cholera. CFTR inhibition therapy may be of particular benefit in young and elderly subjects whose mortality and morbidity remain high despite ORS therapy, as well as in situations where ORS therapy is not available or practical.

Several CFTR inhibitors have been identified, although most of them have some unfavorable pharmacological properties, such as weak potency and lack of CFTR specificity and in vivo efficacy. Previous high-throughput screening using a robust cell-based assay had yielded two classes of CFTR inhibitors, thiazolidinone and glycine hydrazide. The lead compounds for thiazolidinone and glycine hydrazide CFTR inhibitors, CFTRinh-172 and GlyH-101, have been shown to have favorable pharmacological properties, such as an inhibitory mechanism involving direct interaction with the CFTR molecule, reversibility of inhibition, acceptable potency, and in vivo efficacy. However, CFTRinh-172 has poor water solubility (~20 μM) and reduced potency in intact human colonic epithelial cells where its accessibility into the cell is impeded by the interior negative membrane potential. On the other hand, GlyH-101 inhibits calcium-activated chloride channels in addition to CFTR in T84 cells. This finding suggests the need to identify new specific CFTR inhibitors.

Our present studies have revealed two new classes of CFTR inhibitors. Studies in FRT cells showed that INH 1 and INH 2 inhibited cAMP-activated iodide influx and apical chloride secretion in a dose-dependent manner with more than 90% inhibition at concentrations of 100—200 μM. The inhibitory effect of both compounds was abolished within 10—20 min after their removal. Both of them were also effective in inhibiting CFTR-mediated apical chloride current induced by a cell-permeable cAMP analog and a direct CFTR activator, genistein. Taken together, studies in the FRT cells suggest that the results obtained are not due to the cellular toxic effect of the compounds. It also indicates direct inhibitory actions of both compounds on CFTR. However, the possibility that both compounds may indirectly inhibit CFTR via phosphatase activation and PDZ adaptor protein interaction could not be excluded. Thus, the direct interaction of both compounds with CFTR needs to be further examined. Both compounds were also effective in inhibiting cAMP-induced active chloride secretion in human intestinal cells lines, with inhibitory potency comparable to those obtained from apical chloride current measurements in FRT cells. This suggests that the effects of both compounds are cell-type independent and antidiarrheal application of both compounds in human should be possible. However, studies on toxicity and pharmacokinetics of both compounds are still needed to be performed. In addition, further studies on the interaction of these two compounds with CFTR and the possibility of their potential pharmacological benefit in young and elderly subjects whose mortality and morbidity remain high despite ORS therapy, as well as in situations where ORS therapy is not available or practical.

Fig. 5. Effects of CFTR Inhibitors on CaCC Examined by Short-Circuit Current Analysis in T84 Cells

(A) Representative current recordings. In the presence of amiloride (10 μM), CFTRinh-172 (20 μM) and the indicated test chemical (100 μM or 0.1% DMSO) in the apical solution, calcium-activated chloride current was induced by adding carbachol (100 μM) into the basolateral chamber. (B) Summary of results of peak carbachol-induced short-circuit current (mean±S.E., n=3—7). *p<0.01, compared with control value.

Fig. 6. In Vivo Efficacy of CFTR Inhibitors Tested in a Closed Loop Model of Cholera Toxin-Induced Intestinal Fluid Secretion

Intestinal fluid accumulation, shown as loop weight/length (g/cm, S.E.M., n=3—6), was measured at 4 h after injection of cholera toxin. Intestinal loops were injected with PBS with or without cholera toxin (1 μg). Test compounds (3 mg/kg) or DMSO (100 μl, control) was injected intraperitoneally and immediately before abdominal suture was performed. *p<0.01, compared with control value.
needed.

To address the issue of CFTR specificity of the compounds, their effects on calcium-induced intestinal chloride secretion were investigated in T84 cells using carbachol as a Ca\(^{2+}\) agonist. Carbachol, an acetylcholine agonist, binds to a muscarinic receptor (M3) on the basolateral membrane of T84 cells and causes an increase in intracellular calcium through phospholipase C activation. Elevation of intracellular calcium concentration, in turn, allows opening of CaCC via direct gating by calcium and/or a mechanism involving calmodulin-dependent protein kinase CaMKII. Subsequently, transient intestinal chloride secretion occurs. In the present studies, calcium-induced chloride current was unaffected by INH 2, while it was abolished by INH 1. Several studies have demonstrated that chloride channel blockers, including glibenclamide, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and GlyH-101 which inhibit CaCC as well.\(^{7,21—23}\) This suggests a structural homology between CFTR and CaCC. We therefore hypothesize that CaCC may be also a target of INH 1. Actually, off-target effects of a drug can be acceptable as long as it does not disturb normal cellular or physiological functions. Alternatively, it may offer additional benefit, if a gain-of-function of the nonspecific target is known to cause or enhance the disease condition. For example, the observed inhibitory effect on calcium-induced chloride secretion may be of particular value as some forms of diarrhea could result from an enhancement of this secretion process.\(^{14}\) This includes diarrhea that results from infection with rotavirus\(^{24}\) or diarrhea that is associated with the use of aspartyl protease inhibitors,\(^{25}\) an antiviral drug for human immunodeficiency virus-infected patients. Therefore, both cAMP and calcium-activated chloride secretion appear to play a role in the pathogenesis of the infectious secretory diarrhea. Based on this, we consider the inhibitory effect of the compound on calcium-activated intestinal chloride secretion to be advantageous. Furthermore, our in vivo studies showed anti-diarrheal efficacy of INH 1 and suggested that it may be useful in reducing intestinal fluid loss and associated morbidity and mortality in individuals suffering from cholera. In contrast, INH 2 was without antisecretory effect. This may be due to the fact that INH 2 precipitation observed in the mouse abdomen and subsequent inadequate plasma mechanisms of both compounds.

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