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Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker

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Abstract The thiazolidinone $CFTR_{inh}$-172 was identified recently as a potent and selective blocker of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. Here, we characterized the $CFTR_{inh}$-172 inhibition mechanism by patch-clamp and short-circuit analysis using cells stably expressing wild-type and mutant CFTRs. $CFTR_{inh}$-172 did not alter CFTR unitary conductance (8 pS), but reduced open probability by >90% with $K_i \approx 0.6$ μM. This effect was due to increased mean channel closed time without changing mean channel open time. Short-circuit current experiments indicated similar $CFTR_{inh}$-172 inhibitory potency ($K_i \approx 0.5$ μM) for inhibition of Cl^- current in wild-type, G551D, and G1349D CFTR; however, K_i was significantly reduced to 0.2 μM for ΔF508 CFTR. Our studies provide evidence for CFTR inhibition by $CFTR_{inh}$-172 by a mechanism involving altered CFTR gating.

Key words: Cystic fibrosis transmembrane conductance regulator; Chloride channel; Channel blocker; Cystic fibrosis; Chloride secretion

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a plasma membrane Cl^- channel that is involved in the electrolyte/fluid transport in various epithelial cells. Defective CFTR function causes cystic fibrosis (CF), the most common lethal genetic disease in caucasians that produces severe lung disease, pancreatic insufficiency, neonatal intestinal obstruction, and infertility [1]. CFTR is activated by cAMP-dependent phosphorylation at its R domain and modulated by interaction/hydrolysis of ATP at its two nucleotide binding domains NBD-1 and NBD-2 [2].

We recently identified by high-throughput screening a small-molecule CFTR inhibitor, 3-[(3-trifluoromethyl)phenyl]-5-[(3-carboxyphenyl) methylene]-2-thioxo-4-thiazolidinone, labeled $CFTR_{inh}$-172 [3]. $CFTR_{inh}$-172 blocked CFTR-dependent Cl^- currents with $K_i \approx 300$ nM, nearly 500-fold more potent than that of the reference CFTR blocker glibenclamide. Although $CFTR_{inh}$-172 is electrically charged at physiological pH, its block was not affected by changes in membrane potential, suggesting that its binding site does not lie in a region that senses the transmembrane electric field. Unlike other CFTR/Cl^- channel blockers such as diphenylamine-2-carboxylate (DPC), 5-nitro-2(3-phenylpropyl-amino)benzoate (NPPB), niflumic acid, and glibenclamide, which affect other anion and cation channels [4–10], $CFTR_{inh}$-172 was selective at concentrations that strongly inhibit CFTR, without inhibiting Ca^{2+}-dependent and swelling-activated Cl^- channels, or altering ATP-sensitive K^+ channel or MDR-1 function [3]. $CFTR_{inh}$-172 was found to have favorable pharmacological properties in rodents and was effective in blocking intestinal fluid secretion in response to cholera toxin and the heat-stable *Escherichia coli* STa toxin [3,11]. $CFTR_{inh}$-172 is moderately permeable across cell membranes, as shown by its rapid onset of inhibition [3], and across cell layers, as shown from Caco-2 permeability assays [11]. $CFTR_{inh}$-172 has also been proposed to be useful to create animal models of CF.

The mechanism underlying the block of CFTR-dependent Cl^- currents by $CFTR_{inh}$-172 is unknown, possibly involving direct interaction with CFTR at the channel pore, the NBDs, or the R domain. Accordingly, $CFTR_{inh}$-172 may interfere directly with Cl^- flux through the pore or reduce the open channel probability by altering CFTR channel gating. Other CFTR blockers, including glibenclamide and DPC, inhibit Cl^- currents in a voltage-dependent manner, with greater channel inhibition at negative membrane potentials [12–15]. These compounds are thought to bind to a site on the cytosolic side of CFTR and behave as open channel blockers, such that Cl^- flux is interrupted when CFTR is open producing an apparent decrease in mean channel open time. The aim of this study was to elucidate the mechanism of CFTR block using electrophysiological methods. We report evidence for a CFTR inhibition mechanism involving altered channel gating.

2. Materials and methods

2.1. Patch-clamp experiments

Patch-clamp experiments were done at room temperature (22–24°C) on NIH-3T3 fibroblasts stably expressing wild-type CFTR. Cell-attached, inside-out, or whole-cell configurations of the patch-clamp technique were used [16]. The cell membrane was clamped at specified voltages using an EPC-7 patch-clamp amplifier (List Medical). Data were filtered at 250 Hz and digitized at 500 Hz using an ITC-16 data translation interface (Instrutech).

The pipette solution contained (in mM): 120 CsCl, 10 TEA-Cl, 0.5 EGTA, 1 $MgCl_2$, 40 mannitol, 10 Cs-HEPES (pH 7.3). For whole-cell experiments, this solution was supplemented with 0.4 or 3 mM MgATP. The bath solution for cell-attached experiments contained

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(in mM): 130 NaCl, 2 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 20 mannitol, 10 Na-HEPES (pH 7.3). The bath solution for inside-out experiments contained (in mM): 160 CsCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 glucose, 10 Cs-TES, and 0.4 or 1 mM MgATP (pH 7.35). To induce CFTR phosphorylation, this solution was supplemented with 15 μg/ml of the catalytic subunit of protein kinase A (Promega). The bath solution for whole-cell experiments contained (in mM): 150 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 mannitol, 10 Na-TES (pH 7.4). $CFTR_{inh}$-172 was applied in the bath solution at the indicated concentrations. This compound is highly soluble in dimethylsulfoxide, and soluble up to 20–30 μM in physiological saline.

In cell-attached and inside-out experiments, recordings of at least 3 min duration were analyzed. Open channel probability, P_o, was calculated as: $P_o = (t_1+t_2+...t_n)/(Nt_{tot})$, where t_{tot} is the total time, N the number of channels, t_1 the time that one or more channels are open, t_2 the time that two or more channels are open, etc. [17]. Mean channel open time was calculated as described [18]: $t_o = \Sigma j t_j/n$, where t_j is the time that j channels are open at the same time, and n is the total number of open-to-closed state transitions. Mean channel closed time, t_c, was obtained from: $P_o = t_o/(t_c+t_o)$.

For whole-cell and inside-out experiments, membrane potentials are reported using the standard convention with extracellular side of the membrane taken as reference. For cell-attached experiments, the extracellular bath is taken as reference. Preliminary experiments indicated that CFTR currents and block by $CFTR_{inh}$-172 were not affected by membrane potential (not shown). Therefore, cell-attached experiments were generally done at a single pipette potential (+60 mV). Whole-cell membrane currents were recorded by stepping the membrane potential from +80 to −100 mV starting from a holding potential of −5 mV. Inside-out experiments were performed at a membrane potential of −60 mV.

2.2. Short-circuit current

Fischer rat thyroid (FRT) cells expressing wild-type or mutant CFTRs were plated on Snapwell inserts (Corning Costar) as described [3]. After 7–9 days, inserts were mounted in a vertical diffusion chamber (Corning Costar). The basolateral chamber was filled with a solution containing (in mM): 130 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 Na-HEPES (pH 7.3) and 10 glucose. For the apical side, this solution was modified by replacing half of NaCl with Na gluconate and increasing $CaCl_2$ to 2 mM to compensate for calcium buffering caused by gluconate. Both hemichambers were bubbled with air and the temperature maintained at 37°C. The basolateral membrane was permeabilized with 250 μg/ml amphotericin B. Short-circuit current was recorded with a DVC-1000 voltage clamp (World Precision Instruments) using Ag/AgCl electrodes and 1 M KCl agar bridges.

3. Results

Cell-attached patch recordings were performed on NIH-3T3 fibroblasts expressing wild-type CFTR. Little channel activity was detected under resting conditions. Addition of 5 μM forskolin to the bath resulted in multiple channel openings (two to six per patch) having a voltage-independent single-channel conductance of 8 pS as reported previously for CFTR. Addition of $CFTR_{inh}$-172 to the perfusate in the continued presence of forskolin did not change unitary conductance, but reduced channel activity markedly as seen by the less frequent channel openings (Fig. 1A) and the all-point histograms (Fig. 1A, right). Fig. 1B summarizes open channel probability (P_o) measured at different $CFTR_{inh}$-172 concentrations, showing a dose-dependent decrease in P_o with K_i of 0.57 μM and Hill coefficient, n_H, of 0.83.

To clarify the basis for the change in P_o, mean channel open and closed times were measured at different $CFTR_{inh}$-172 concentrations (Fig. 1C). Interestingly, mean channel open time was not changed whereas the mean channel closed time was significantly prolonged in a dose-dependent manner. At 2 μM $CFTR_{inh}$-172 mean channel closed time increased

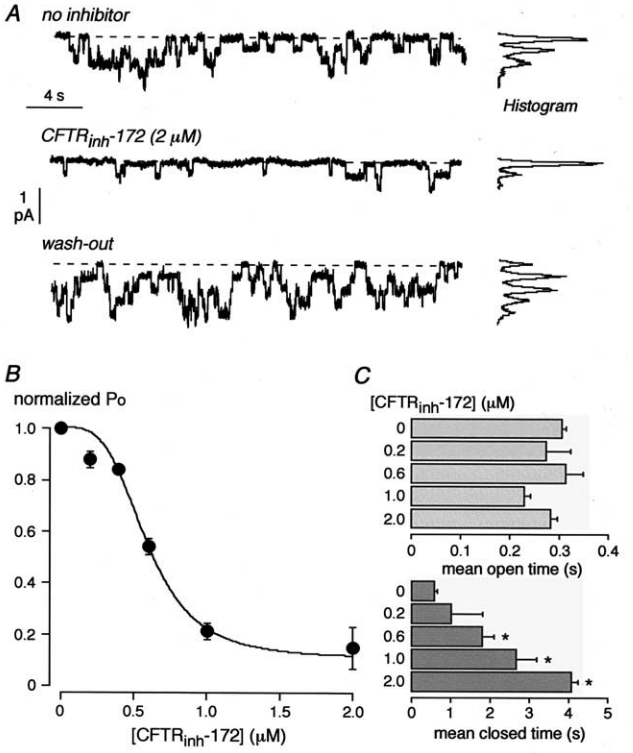

Fig. 1. Block of CFTR currents in cell-attached patches. A: Representative recordings from a cell-attached patch-clamp experiment showing CFTR channel activity with and without $CFTR_{inh}$-172 in the bath (extracellular) solution. Dashed lines show zero current level (channels closed) with downward deflections indicating channel openings. Pipette potential was +60 mV. All-point histograms are shown on the right of each trace. Note the decrease in the areas of peaks corresponding to multiple channel levels. B: Open channel probability (P_o) versus inhibitor concentration with fitted K_i 0.57 ± 0.06 μM and Hill coefficient 0.83 ± 0.07. C: Mean channel open (top) and closed (bottom) times versus $CFTR_{inh}$-172 concentration (± S.E.M., n = 3–5). Asterisks indicate a significant difference ($P <$ 0.01) compared to no inhibitor.

from 0.6 to 4.2 s whereas mean channel open time remained ~0.3 s. CFTR is characterized by two different closure events: brief intraburst closures and long-lasting interburst closures. When the results were reanalyzed by ignoring the closures below 30 ms, $CFTR_{inh}$-172 still increased closed time without affecting open time. For example, t_c increased from 3.3 ± 0.3 to 17 ± 2 s in the presence of 2 μM $CFTR_{inh}$-172 ($P < 0.01$; not shown), whereas t_o did not change significantly (1.3 ± 0.1 vs. 1.1 ± 0.2). Thus $CFTR_{inh}$-172 inhibits CFTR by an altered channel gating mechanism.

Similar measurements were done using the known (though relatively less potent and specific) CFTR blocker glibenclamide. At 100 μM, a glibenclamide concentration that partially blocked CFTR, frequent fast closures were seen, with a > 10-fold decrease in apparent mean open time (Fig. 2). This behavior contrasts sharply with the $CFTR_{inh}$-172 inhibition mechanism.

Cell-detached patch-clamp experiments were done using the inside-out configuration to assess $CFTR_{inh}$-172 efficacy under cell-free conditions and to test whether ATP is competitive with the $CFTR_{inh}$-172 block. After patch excision, channel openings were not observed prior to CFTR activation (Fig. 3A). Multiple channel openings were seen after bath (cytosolic

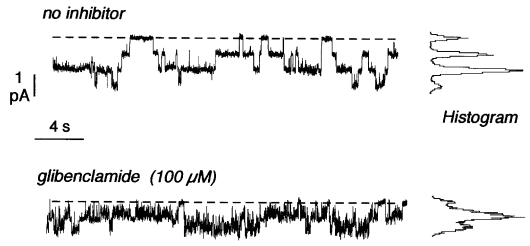

Fig. 2. Glibenclamide inhibition of CFTR. Representative cell-attached experiment showing the effect of extracellular perfusion with 100 μM glibenclamide. In contrast to $CFTR_{inh}$-172, glibenclamide caused a marked increase in the frequency of brief closures that interrupt the bursts of channel openings. Apparent mean open time decreased from 573 to 48 ms. Pipette potential was +60 mV.

side) application of the catalytic subunit of protein kinase A and ATP. Addition of $CFTR_{inh}$-172 produced a reversible decrease in channel activity similar to that observed in cell-attached recordings. Additional inside-out patch-clamp experiments were done to test for ATP competition using two concentrations of $CFTR_{inh}$-172 (0.2 and 1 μM) and ATP (0.4 or 1 mM) in the bath (cytosolic) solution. The decrease in P_o caused by $CFTR_{inh}$-172 was not dependent on ATP concentration (Fig. 3B). Similar results were obtained using the whole-cell patch-clamp configuration using specified pipette solutions to set cytoplasmic ATP concentration. The dose response for $CFTR_{inh}$-172 inhibition of CFTR Cl^- current was not different at 0.4 and 3 mM concentrations of ATP in the pipette solution (Fig. 3C). These results indicate that $CFTR_{inh}$-172 interacts directly with CFTR, and that $CFTR_{inh}$-172 and ATP do not compete.

To test whether $CFTR_{inh}$-172 interacts with one of the NBDs, we compared the inhibitory potency of $CFTR_{inh}$-172 on wild-type CFTR and the mutants G551D and G1349D that produce defective CFTR gating NBD-1 and NBD-2, respectively. Transfected FRT cells were studied in short-circuit current experiments. After activating wild-type CFTR using a high concentration of cpt-cAMP, $CFTR_{inh}$-172 blocked the resulting Cl^- current in a dose-dependent manner, with a K_i of 0.37 μM ($n_H = 0.92$) (Fig. 4). The flavone genistein (at 200 μM) together with cpt-cAMP was used to maximally activate the CFTR mutants G551D and G1349D. $CFTR_{inh}$-172 blocked G551D and G1349D Cl^- currents with potency not significantly different from that for inhibition of wild-type CFTR, with K_i of 0.53 μM ($n_H = 0.90$) and 0.51 ($n_H = 1.17$), respectively, for G551D and G1349D (Fig. 4B,C). To test

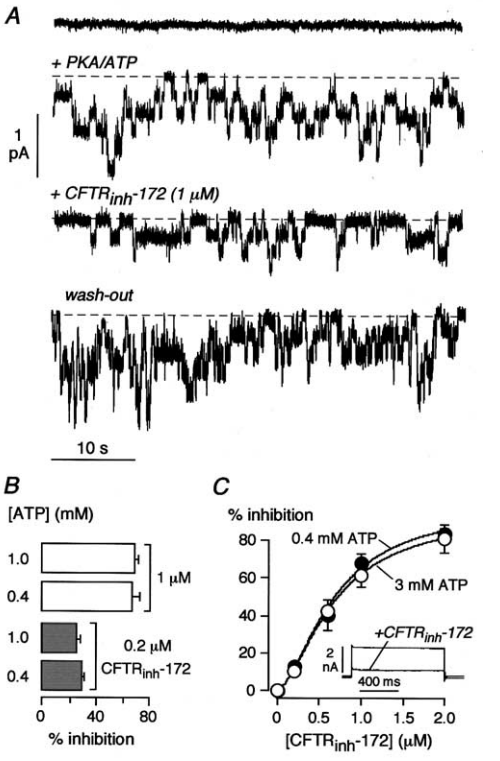

Fig. 3. $CFTR_{inh}$-172 inhibition in excised membrane patches and ATP dependence. A: Representative traces from an excised inside-out patch showing no basal activity, multiple channel openings after bath (cytosolic) application of catalytic subunit of protein kinase A (15 μg/ml) and ATP (1 mM), decreased channel activity after $CFTR_{inh}$-172, and recovery after inhibitor wash-out. B: Inhibition of open channel probability in inside-out patches as a function of $CFTR_{inh}$-172 concentration (0.2 and 1 μM) and ATP concentration (0.4 and 1 mM). Differences not significant. C: Dose responses for $CFTR_{inh}$-172 inhibition in whole-cell experiments with 0.4 or 3 mM ATP in the pipette (intracellular solution) and membrane potential clamped at +80 mV (S.E.M., $n = 4$–5). Inset: Representative whole-cell membrane currents elicited at +80 mV with and without $CFTR_{inh}$-172. The same dose response was obtained by clamping the membrane potential at −100 mV (not shown).

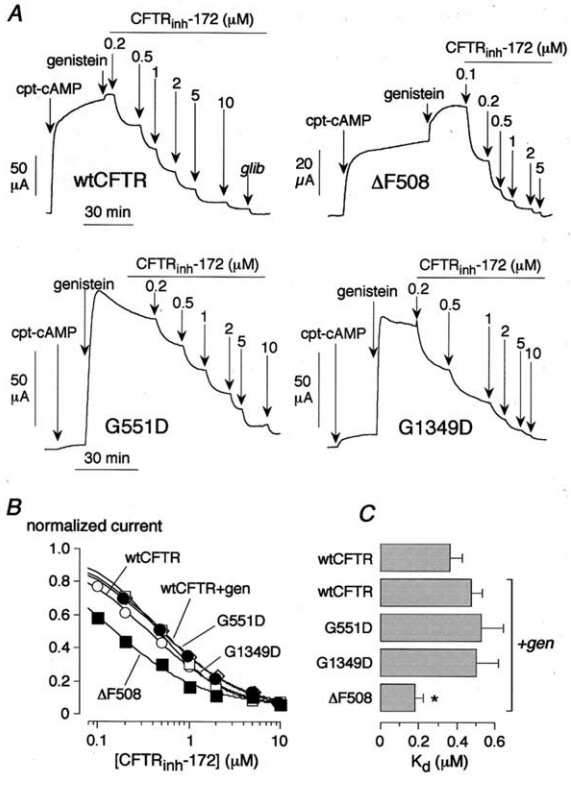

Fig. 4. Sensitivity of CFTR mutants to inhibition by $CFTR_{inh}$-172. A: Representative short-circuit current experiments on permeabilized FRT cells expressing wild-type CFTR, or G551D, G1349D, and ΔF508 mutants. cpt-cAMP concentration was 100 μM and genistein concentrations were 50 μM (wild-type and ΔF508) or 200 μM (G551D and G1349D). Cells expressing ΔF508-CFTR were incubated at 27°C for 24 h prior to measurements to rescue the mutant protein from the endoplasmic reticulum [19]. B: $CFTR_{inh}$-172 dose–response curves. C: K_d obtained by fitting dose–response curves (S.E.M., $n = 5$–8). $^*P < 0.01$ and 0.05 compared to wild-type CFTR with and without genistein, respectively.

whether CFTR activation by genistein could alter $CFTR_{inh}$-172 potency, a $CFTR_{inh}$-172 dose response was done on wild-type CFTR after activation with 50 μM genistein (the maximal possible concentration on the wild-type protein since higher concentrations induce inhibition). After genistein activation, the $CFTR_{inh}$-172 inhibitory potency did not differ significantly ($K_i = 0.48$; $n_H = 0.96$) from that measured for G551D and G1349D. Thus, these gating mutants were not informative in defining the location of the putative $CFTR_{inh}$-172 binding site on CFTR. Therefore, we also tested the efficacy of $CFTR_{inh}$-172 in cells expressing ΔF508-CFTR (after low temperature rescue [19]), the most frequent mutation in CF subjects. Interestingly, ΔF508-CFTR Cl^- currents were inhibited by $CFTR_{inh}$-172 with a significant about two-fold greater efficacy than found for wild-type CFTR and the G551D and G1349D mutants (Fig. 4B,C). This finding suggests $CFTR_{inh}$-172 interaction at the CFTR NBD-1 domain.

4. Discussion

The purpose of this study was to define the CFTR inhibition mechanism of the thiazolidinone $CFTR_{inh}$-172, a small molecule CFTR inhibitor identified recently by high-throughput screening of a collection of diverse small molecules [3]. $CFTR_{inh}$-172 was substantially more potent and CFTR-selective than the existing Cl^- channel blockers glibenclamide, NPPB, niflumic acid, and DPC. We reported previously that $CFTR_{inh}$-172 strongly blocked CFTR currents in Ussing chamber and whole-cell patch-clamp experiments, and that the $CFTR_{inh}$-172 block was not affected by the applied membrane potential [3]. However, the mechanism of $CFTR_{inh}$-172 block was not characterized, nor was its possible site of interaction on the CFTR molecule.

We found here in cell-attached recordings that $CFTR_{inh}$-172 strongly reduced open channel probability without affecting single channel conductance. Interestingly, $CFTR_{inh}$-172 increased the time that the channel spends in the closed state without affecting mean open time. This inhibition mechanism contrasts with other known CFTR blockers, including glibenclamide and DPC, which are open channel blockers [12–15]. Open channel blockers decrease mean channel open time by a mechanism that may involve plugging into the channel pore and interrupting anion flux. The increase in mean CFTR channel closed time after $CFTR_{inh}$-172 indicates modification of channel gating rather than block of the channel conductive pathway. This conclusion is in agreement with the finding that $CFTR_{inh}$-172 block, in contrast to glibenclamide and DPC [12–15], is not affected by the transmembrane voltage despite its negative charge. Therefore, the $CFTR_{inh}$-172 binding site probably does not lie close to the pore in a region sensitive to the transmembrane electrical field, bur rather in a structure that controls channel gating. The alteration of CFTR gating by $CFTR_{inh}$-172 resembles that described for genistein [17,18]. Although at low micromolar concentrations genistein is a CFTR activator, at high micromolar concentrations genistein decreases P_o due to a prolongation of the closed time. Changes in CFTR mean closed time have also been reported for electrolyte-dependent variations in P_o [20].

One hypothesis to explain $CFTR_{inh}$-172 inhibition is impairment of CFTR channel gating by binding to one of the NBDs, possibly by interfering with ATP/NBD interactions. However, in inside-out and whole-cell patch-clamp experiments there was no effect of ATP concentration on the efficacy of the $CFTR_{inh}$-172 block thus indicating that the thiazolidinone blocker does not compete with ATP for binding to either NBD.

We also tested the effect of CFTR mutations that impair NBD function and thus reduce CFTR opening, including G551D and G1349D. Such mutations are localized in NBD-1 and NBD-2, respectively, and have been shown to impair ATP binding [21,22]. $CFTR_{inh}$-172 had comparable inhibitory potency for reducing Cl^- currents for wild-type CFTR, G551D, and G1349D. This finding supports the conclusion that $CFTR_{inh}$-172 does not act by interfering with ATP binding at CFTR NBDs. However, remarkably, the $CFTR_{inh}$-172 K_i was significantly reduced for the CFTR mutant ΔF508, suggesting that $CFTR_{inh}$-172 may interact with a specific site on the NBD-1 domain of CFTR where the ΔF508 mutation resides, though allosteric effects on other protein domains cannot be excluded. It has been reported that the ΔF508 protein has a decreased open channel probability due to longer permanence in a closed state [23,24]. Therefore, the ΔF508 mutant has a defective conformation and function also when targeted to the plasma membrane by cell incubation at low temperature. It is possible that $CFTR_{inh}$-172 has greater affinity to the specific protein conformation generated by the ΔF508 mutation.

In conclusion, our study shows that the thiazolidinone $CFTR_{inh}$-172 behaves differently from other known blockers of the CFTR channel. Rather than blocking the channel pore, it affects the mechanism of channel gating, possibly by binding to NBD-1. In addition to its potential applications as an antidiarrheal agent and in creating the CF phenotype, $CFTR_{inh}$-172 could thus be a useful tool to investigate the molecular mechanisms underlying CFTR function and regulation.

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