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Thiazolidinone CFTR inhibitors with improved water solubility identified by structure–activity analysis

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ABSTRACT

The thiazolidinone 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone ($CFTR_{inh}$-172) inhibits cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel conductance with submicromolar affinity and blocks cholera toxin-induced intestinal fluid secretion. Fifty-eight $CFTR_{inh}$-172 analogs were synthesized to identify CFTR inhibitors with improved water solubility, exploring modifications in its two phenyl rings, thiazolidinone core, and core-phenyl connectors. Greatest CFTR inhibition potency was found for 3-CF_3 and polar group-substituted-phenyl rings, and a thiazolidinone core. Two compounds with ~1 μM CFTR inhibition potency and solubility >180 μM (>10-fold more than $CFTR_{inh}$-172) were identified: Tetrazolo-172, containing 4-tetrazolophenyl in place of 4-carboxyphenyl, and Oxo-172, containing thiazolidinedione in place of the thiazolidinone core. These water soluble thiazolidinone analogs had low cellular toxicity. The improved water solubility of Tetrazolo- and Oxo-172 make them potential lead candidates for therapy of secretory diarrheas and polycystic kidney disease.

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride channel, which when mutated can produce the hereditary disease cystic fibrosis. CFTR inhibition is a potential strategy for therapy of secretory diarrheas[1–6] and polycystic kidney disease,[7–9] as well as for pharmacological creation of the cystic fibrosis phenotype in human and animal tissues.[10] We previously discovered by high-throughput screening the thiazolidione CFTR inhibitor 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone ($CFTR_{inh}$-172), which blocked CFTR chloride conductance fully with IC_{50} < 0.5 μM.[11] Patch-clamp analysis indicated a voltage-independent channel block mechanism with prolongation of mean channel closed time,[12] involving interaction at arginine-347 located near the CFTR cytoplasmic surface.[13] In vivo analysis indicated $CFTR_{inh}$-172 efficacy in reducing cholera toxin-induced intestinal fluid secretion in rodent models.[11,14,15] $CFTR_{inh}$-172 was found to have low toxicity, renal excretion with minimal metabolism, and intestinal accumulation by enterohepatic recirculation.[14] $CFTR_{inh}$-172 has been used extensively to block CFTR chloride channel function in variety of cell culture, tissue, and in vivo systems.[16–23]

Limitations of $CFTR_{inh}$-172 include its low water solubility and oral bioavailability. $CFTR_{inh}$-172 is a weak acid with a single negative charge at neutral pH, which precipitates in an acid environment. Also, the concentration of $CFTR_{inh}$-172 in cytoplasm, where it acts, is likely reduced in a Nernstian manner compared with its extracellular concentration because of the interior-negative membrane potential of its target epithelial cells. We report here a structure–activity analysis of $CFTR_{inh}$-172, with the goal of improving water solubility while maintaining CFTR inhibition potency. We systematically modified Rings A and C of $CFTR_{inh}$-172, as well as the thiazolidinone core (Ring B) and the core-ring linkers (Fig. 1). CFTR analogs with >10-fold higher water solubility than $CFTR_{inh}$-172 and only mildly reduced potency were synthesized and characterized.

2. Results

2.1. Chemistry

A total of 58 $CFTR_{inh}$-172 analogs were assayed for CFTR inhibition activity, with the most potent compounds further characterized. Of the 58 compounds, several (compounds **5**, **6**, **9**, **14**, **15**, **17**, **20**, **21**, **25**, **26**, **27**, **29**, **33**, and **38**) were reported previously as part of other studies from our laboratory.[9,11,13,14]

Compounds with different substituents on Ring A were synthesized, keeping the remainder of the molecule the same. Commercially available substituted anilines **1** were reacted with carbon disulfide, followed by reaction with bromoacetate and acidic cyclization to give thiazolidinone intermediate **3** (Route 1, Scheme 1).

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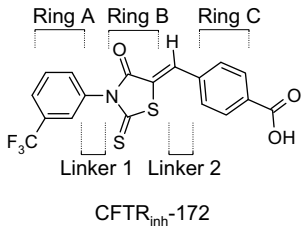


Figure 1. Chemical structure of $CFTR_{inh}$-172 showing Rings A, B, C and Linkers 1 and 2.

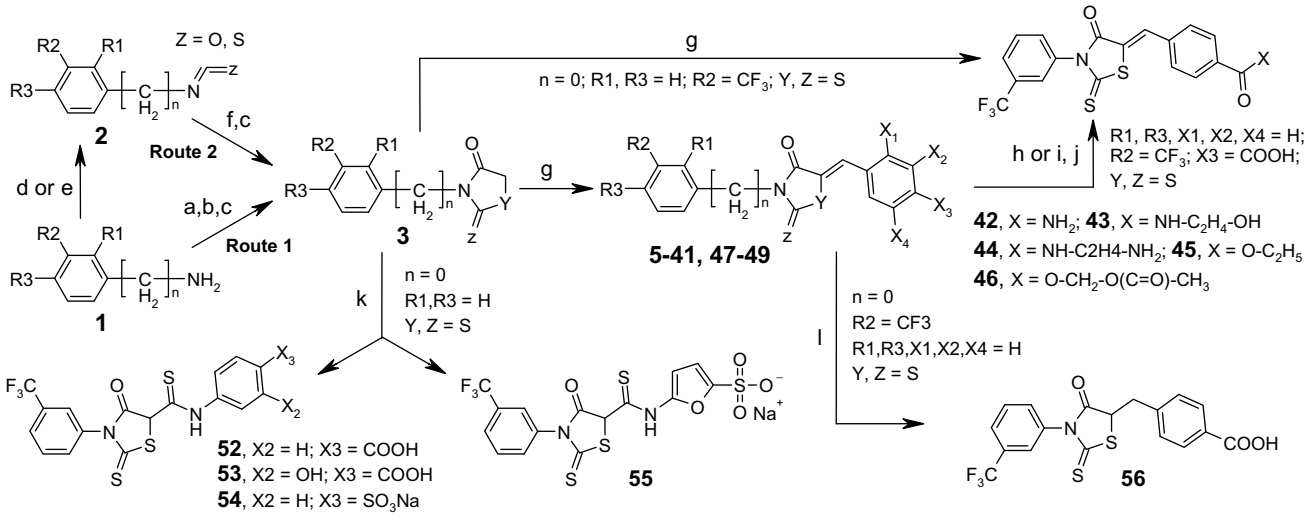
The same reaction route was used for synthesis of analogs containing a methylene group between Rings A and B (Route 1, Scheme 1). These intermediates upon Knoevenagel condensation with aromatic aldehydes in ethanol under reflux in presence of piperidine produced analogs **5**–**41** and **47**–**49**. TLC showed quantitative formation of products. This reaction generates a double bond that can produce E and Z isomers. Similar analogs are reported to exist predominantly as Z-isomers.[24–29] It is presumed that the analogs synthesized here are mainly Z-isomers.

Scheme 1 also shows an alternate isothiocyanate route (Route 2) for synthesis of the 2-thiaoxo-4-thiazolidinone ring intermediate **3**. Route 2 was used for the synthesis of **13**, **18**, **20**, **22**, and **27**. Isothiocyanates **2** were prepared by single step reaction of corresponding amino compounds **1** with thiophosgene and reacted with thioglycolic acid in presence of triethylamine to yield dithiocarbamate intermediates. This intermediate upon in situ acidification (HCl) and reflux generated 2-thioxo-4-thiazolidinones **3**. Route 2 was single-pot and increased overall yields compared to Route 1, though use of toxic compounds (phosgene or thiophosgene) was required.

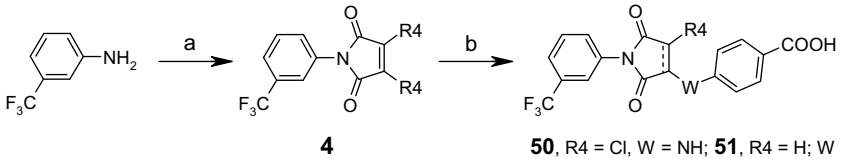
Carboxylate modification to various esters and amides was achieved by three reaction routes as shown in Scheme 1. Amide **42** and ester **45** were synthesized by condensation of thiazolidinone intermediate **3** with 4-carbamoylbenzaldehyde and ethyl 4-formylbenzoate, respectively. Alternatively, the carboxyl in $CFTR_{inh}$-172 was converted to the acid chloride using thionyl chloride, followed by reaction with equimolar amounts of compounds (ammonia, aminoethanol, ethylenediamine, acetoxymethanol) to yield **42**–**44** and **46**. Reaction involving activation of the carboxy function by DCC also generated **42**–**44** and **46** amides (Scheme 1).

Thiazolidinedione **48** was synthesized by condensation of 2,4-thiazolidinedione intermediate **3** (R1, R3 = H, R2 = CF_3, Y = S, Z = O) with 4-carboxybenzaldehyde (Scheme 1). Route 2 was used for efficient synthesis of corresponding intermediate **3**. For synthesis of compounds **50** and **51**, maleimide intermediates **4** (R4 = Cl or H) were prepared by reaction of 3-trifluoromethylaniline with dichloromaleic anhydride (R4 = Cl) or maleic anhydride (R4 = H) in refluxing acetic anhydride (Scheme 2). Subsequent reaction with 4-aminobenzoic acid and 4-mercaptobenzoic acid produced compounds **50** and **51** (Scheme 2, dotted line indicate double bond in **50**).[30–33] Compounds **52**–**55** were synthesized by reaction of aryl isothiocyanates with **3** in presence of base DBU at room temperature (Scheme 1). Reduction of the double bond in $CFTR_{inh}$-172 using $LiBH_4$ in pyridine[24,34] at room temperature gave **56** (Scheme 1).

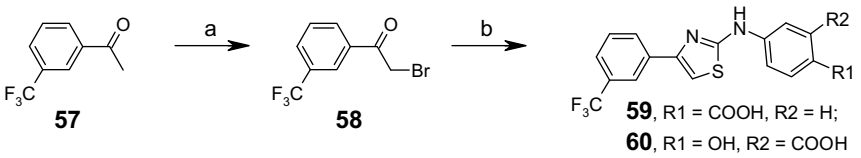
Thiazole analogs **59** and **60** were synthesized by bromination of acetophenone **57** in acetic acid at 0 °C for 2 h, followed by reaction with substituted phenylthiourea in refluxing ethanol (Scheme 3).[35,36] Synthesis of thiadiazole compounds **64** and **65** was accom-



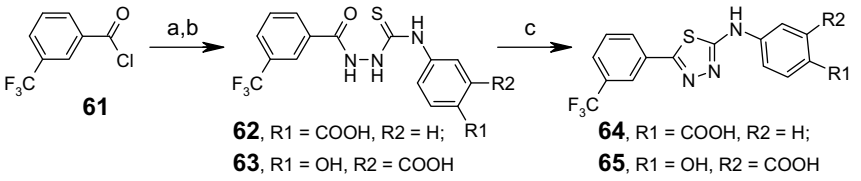
Scheme 1. Reagents and conditions: (a) CS_2, TEA, EtOAc, rt, 12 h; (b) $BrCH_2COOH$, $NaHCO_3$, rt, 2 h; (c) HCl, reflux, 2 h; (d) $COCl_2$ or $CSCl_2$, TEA, 10 °C, 2 h; (e) Triphosgene, $CHCl_3$, reflux, 3 h; (f) $HSCH_2COOH$, TEA, 4 h. (g) aldehyde, piperidine/ethanol, reflux, 2 h.; (h) $SOCl_2$, cat. DMF, rt, 2 h; (i) DCC, *N*-hydroxy-succinimide, rt,; (j) HX-R, pyridine, 0°-rt, 2 h; (k) SCN-($3X_2$–$4X_3$–Ph) or sodium 5-SCN-furan-2-sulfonate (for **55**), DBU, THF, rt, 2 h. (l) $LiBH_4$, pyridine, rt, 12 h.



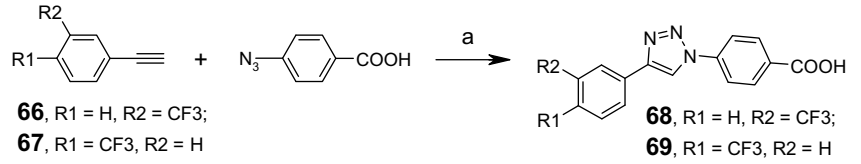
Scheme 2. Reagents and conditions: (a) malic anhydride/dichloromalic anhydride, Ac_2O, NaOAc, 80°, 2 h; (b) (4-COOH)–Ph–WH, TEA, THF, rt, 5 h.



Scheme 3. Reagents and conditions: (a) Br_2, AcOH, 0°, rt, 2 h; (b) *n*-(4-carboxyphenyl)thiourea, AcOH, reflux, 4 h.



Scheme 4. Reagents and conditions: (a) $NH_2NH_2 \cdot xH_2O$, pyridine, rt, 2 h; (b) (4-COOH)–Ph–NCS, TEA, THF, rt, 12 h; (c) H_2SO_4, rt, 2 h.



Scheme 5. Reagents and conditions: (a) 1 mol% $CuSO_4$, 0.1 eq. sodium ascorbate, H_2O/*t*-BuOH (1:1), rt, 24 h.

plished in three steps. Reaction of the acid hydrazide (prepared from benzoyl chloride **61** and hydrazine) with substituted phenylisothiocyanates gave thiosemicarbazides **62** and **63** in good yields. Treatment of **62** and **63** with sulfuric acid produced the 2-aminothiadiazoles **64** and **65** (Scheme 4).[37]

Scheme 5 shows the synthesis of 1,2,3-triazoles. 1,3-Dipolar cycloaddition[38,39] of alkynes **66** and **67** with 4-azidobenzoic acid produced the 1,2,3-triazoles (compounds **68** and **69**) in high yields. Single spots in TLC indicated that adducts were predominantly 4-regioisomers.

2.2. Functional studies

CFTR inhibition potency for all compounds was measured by short-circuit current analysis, with representative original data and concentration–inhibition curves for compounds of greatest interest provided in Figure 2. In the short-circuit current assay, CFTR was stimulated by the cAMP agonist forskolin, followed by compound additions at increasing concentration. Table 1 summarizes the effects of Ring A substitutions on CFTR inhibition potency. CF_3 substitution at the 3-position, as in $CFTR_{inh}$-172, gave greater inhibition potency than at the 2- or 4-position (compounds **20** and **25**).[9] Substitution of 3-CF_3 by 3-CH_3 gave the less active analog **12**. Compounds **8** and **11**, in which Ring A contained 3-CF_3 along with 4-F substituents, had intermediate activity. Similar substitutions by chloro at 2- or 4-position greatly reduced activity (compounds **24** and **30**).

To improve aqueous solubility, we first tried to disrupt the planar configuration of Rings A and B, which is predicted to reduce ring-ring stacking, by adding bulky ortho substituents to Ring A. Introduction of methyl at the 2-position in α-Me-172, **18**, increased water solubility, but reduced inhibition potency considerably. Figure 2 shows concentration-dependent inhibition of CFTR by $CFTR_{inh}$-172 (*A*) and α-Me-172 (*E*) with IC_{50} 0.4 and 8 μM, respectively. As determined by LC/MS, the solubility of α-Me-172 in saline was 259 μM, substantially greater than that of $CFTR_{inh}$-172 (17 μM; Table 3). Addition of polar substituents such as hydroxy, SO_3Na or COOH in Ring A, or removal of CF_3, produced highly water-soluble though inactive compounds.

The thiazolidinone core, Ring B, was replaced by thiazolidinedione, maleimide, succinimide, thiazole, thiadiazole, and triazole, while keeping Rings A and C and their substituents the same as in $CFTR_{inh}$-172. These heterocycles were selected because of their resemblance to Ring B, their ease of synthesis, and prior knowledge of good bioavailability and metabolic and chemical stability.[40,41]

The 2,4-thiazolidinedione **48** is a close analog of $CFTR_{inh}$-172 in which the thioxo group is replaced by an oxo-group (referred to as Oxo-172). Replacement of 2-thioxo by 2-oxo increased solubility in saline by ~25-fold, while reducing CFTR inhibition potency 3.6-fold in short-circuit current assays (Fig. 2B and F; Table 2). Maleimide analog **50** had weak activity, though 2,5-pyrrolidinedione **51** had moderate activity with $IC_{50} \sim 7$ μM. The Ring B variation in **51** removed the double bond in $CFTR_{inh}$-172, making it less reactive for Michael addition. The aminothiazole and aminothiadiazole analogs were inactive, however. Replacement of thiazolidinone by other heterocycles such as aminothiazoles (**59** and **60**) gave weakly active compounds. Replacement of thiazolidinone by aminothiadiazoles (**64** and **65**), and 1,2,3-triazoles (**68** and **69**) yielded inactive compounds.

C-ring substitutions were carried out in an attempt to increase compound solubility, and to convert compound net negative charge at physiological pH to neutral in order to increase compound accumulation in cytoplasm. Analogs were synthesized with different substitutions on Ring C, keeping Rings A and B, and both linkers the same as in $CFTR_{inh}$-172. Substitutions were chosen to increase compound polarity and H-bond capacity, including carboxy, esters, amides, hydroxy, methoxy, and sulfonate.

Monosubstituted compounds containing 4-COOH ($CFTR_{inh}$-172) or 3-COOH, **9**, showed greater CFTR inhibition potency than 2-COOH, **26**. Esterification or amidation of 4-COOH in $CFTR_{inh}$-172 gave inactive compounds **42**–**46**. Compounds **31**, **38**, **19**, and **34** containing mono, di-, or tri-hydroxy functions at Ring C had low

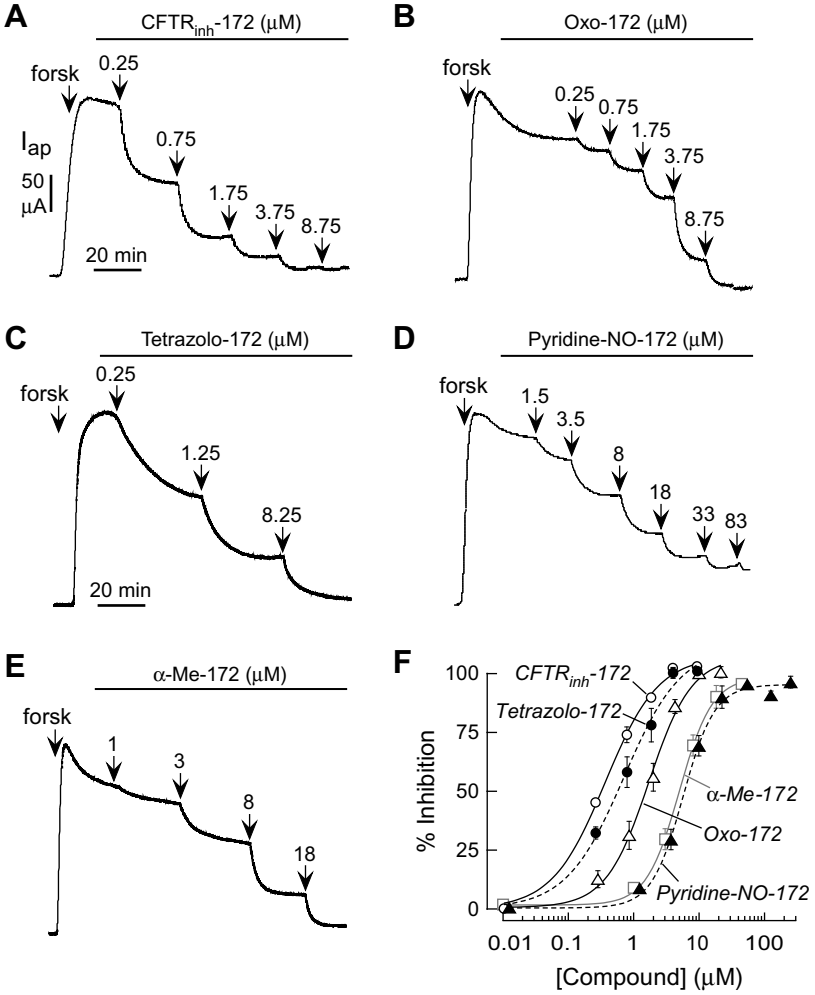


Figure 2. Short-circuit current measurements of CFTR inhibition. (A) CFTR-mediated apical membrane chloride current measured in FRT cells expressing human wildtype CFTR after permeabilization of the basolateral membrane in the presence of a chloride gradient (see Section 4). CFTR was activated by 20 μM forskolin and indicated concentrations of inhibitors were added. (B) Concentration–inhibition data for $CFTR_{inh}$-172, Tetrazolo-172, Oxo-172, α-Me-172, and Pyridine-NO-172. See Table 3 for fitted IC_{50} values.

activity. Based on predicted pK_a of >7.5, these compounds are expected to be neutral at physiological pH. Creation of a negative charge by addition of 3,4-dibromo electron-withdrawing moieties, that lower the pK_a of 4-OH, resulted in modest inhibition activity (compounds **14** and **15**; Table 1), whereas modification of the ionizable 4-OH to 4-OMe gave the inactive compound **39**. However, sulfonic acid derivatives **35** and **37**, which carry at physiological pH single and double negative charges, respectively, were inactive.

Ring C was also replaced by heterocyclic-equivalent ring systems. Replacement of the phenyl by a pyridyl ring gave the inactive neutral compound **33**; however, N-oxidation of pyridyl nitrogen gave the first, net-neutral thiazolidinone CFTR inhibitor, pyridine-NO-172, **17**, with IC_{50} 9 μM (Fig. 2D and F). This is a zwitterionic compound containing a positive charge on the ring nitrogen and negative charge on the oxygen. Addition of hetero atoms is expected to increase aqueous solubility by H-bonding and increased polarity. Pyridine-NO-172 had high aqueous solubility of 264 μM. Similarly, the polar analog **10** containing a 4-carboxymethoxy group had an IC_{50} of 2.6 μM (Table 1). Moving the carboxymethoxy group from the 4-position (as in **10**) to the 3-postion (as in **36**) substantially reduced CFTR inhibition.

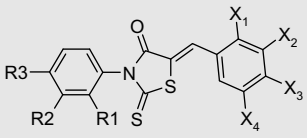
As another approach to improve water solubility, the 4-COOH in ring C was replaced by tetrazolo-5-yl, an isoster of carboxy with delocalized negative charge at physiological pH. The tetrazolo substitution increased water solubility 11-fold (Table 3) with IC_{50} of 0.8 μM (Fig. 2F). As shown in Figure 2C, Tetrazolo-172 showed slower inhibition kinetics than $CFTR_{inh}$-172 or Oxo-172. Similarly replacement of carboxylate in **48** by tetrazolo in **49** gave lower inhibition potency (IC_{50} 17 μM; Table 2).

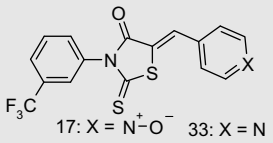
$CFTR_{inh}$-172 contains a single bond as a Linker 1 that directly connects lipophilic Ring A to heterocyclic Ring B. We examined the effect of introduction of a methylene group as a bridge between Rings A and B. Compound **47** was inactive, however, indicating little tolerance of Linker 1 modification.

Because the double bond in Linker 2 is a Michael electrophile, alternative linkers between Ring B and Ring C were investigated. First, reduction of double bond linker of $CFTR_{inh}$-172 gave compound **56**, which was inactive. The double bond reduction disturbes the rigid geometry of $CFTR_{inh}$-172, which is presumed as Z-configuration, allowing free rotation of Rings B and C. Further, replacement of the double bond-methylidyne bridge by thioamide in **52** and **53**, though highly water soluble, were inactive, as were analogs **54** and **55** containing sulfonic acid substituents (Scheme 1 and Table 2).

Preliminary cytotoxicity analysis was done for the principle compounds of interest, Tetrazolo-172, Oxo-172, α-Me-172, and Pyridine-NO-172, comparing with $CFTR_{inh}$-172 (Table 3). Compounds were incubated with cell cultures for 48 h and cell viability

Table 1
Variations in Rings A and C





Compound	R3	R2	R1	X1	X2	X3	X4	IC_{50} (μM)
5 $CFTR_{inh}$-172	H	CF_3	H	H	H	COOH	H	0.4
6 Tetrazolo-172	H	CF_3	H	H	H	Tetrazolo	H	0.8
7	H	CF_3	H	H	COOH	OH	H	1.4
8	F	CF_3	H	H	H	COOH	H	1.5
9	H	CF_3	H	H	COOH	H	H	1.5
10	H	CF_3	H	H	H	O–CH_2–COOH	H	2.6
11	F	CF_3	H	H	COOH	H	H	5
12	H	CH_3	H	H	H	COOH	H	5
13	H	CH_3	CH_3	H	H	COOH	H	7
14	H	CF_3	H	H	Br	OH	Br	7
15	H	CF_3	H	OH	Br	OH	Br	8
16	H	CF_3	H	OH	H	COOH	H	
17 Pyridine-NO-172								9
18 α-Me-172	H	CF_3	CH_3	H	H	COOH	H	9
19	H	CF_3	H	H	OH	OH	OH	11
20	H	H	CF_3	H	H	COOH	H	12
21	CF_3	H	H	H	COOH	H	H	13
22	H	H	CF_3	H	COOH	OH	H	14
23	Ring A: 3,5-di-CF_3–Ph–			H	H	COOH	H	14
24	Cl	CF_3	H	H	H	COOH	H	15
25	CF_3	H	H	H	H	COOH	H	15
26	H	CF_3	H	COOH	H	H	H	15
27	H	H	CF_3	COOH	H	H	H	18
28	H	H	CF_3	H	COOH	H	H	19
29	CF_3	H	H	COOH	H	H	H	20
30	Ring A: 2-Cl-5-CF_3–Ph–			H	H	COOH	H	20
31	H	CF_3	H	H	H	OH	H	24
32	CF_3	H	H	H	COOH	OH	H	25
33	–							28
34	H	CF_3	H	OH	OH	OH	H	28
35	H	CF_3	H	H	H	SO_3Na	H	29
36	H	CF_3	H	H	O–CH_2–COOH	H	H	30
37	H	CF_3	H	SO_3Na	H	SO_3Na	H	50
38	H	CF_3	H	H	OH	OH	H	Inactive
39	H	CF_3	H	OH	Br	OMe	Br	Inactive
40	H	CF_3	H	OCH_3	H	Br	H	Inactive
41	H	CF_3	H	H	H	OMe	H	Inactive
42	H	CF_3	H	H	H	$CONH_2$	H	Inactive
43	H	CF_3	H	H	H	$CONHC_2H_4OH$	H	Inactive
44	H	CF_3	H	H	H	$CONHC_2H_4NH_2$	H	Inactive
45	H	CF_3	H	H	H	$COOCH_2CH_3$	H	Inactive
46	H	CF_3	H	H	H	$COOCH_2OCOCH_3$	H	Inactive

was determined by crystal violet staining. Compounds at 20 μM showed little cytotoxicity, with staining ~90% of that of control (vehicle-treated cultures). Crystal violet staining was reduced for Tetrazolo-172 and α-Me-172 at 50 μM. $CFTR_{inh}$-172 was not studied at 50 μM because of its limited aqueous solubility.

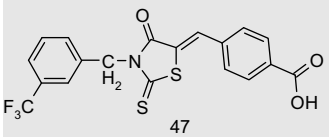
3. Discussion

We synthesized and characterized 58 $CFTR_{inh}$-172 analogs with the goal to increase water solubility and retain CFTR inhibition potency. As summarized in Figure 3, SAR analysis revealed that compounds containing 3-CF_3 on Ring A, thiazolidinone core as Ring B, and 4-carboxy at Ring C had greatest CFTR inhibition potency. Polar ionizable substituents such as carboxy, hydroxy, tetrazolo, which created a negative charge on Ring C, gave greatest inhibition activity, whereas non-polar substituents were required on Ring A. The thiazolidinone ring (Ring B) could be replaced by thiazolidinedione, and the COOH group on Ring C by tetrazolo, yielding compounds with greatly increased water solubility compared to $CFTR_{inh}$-172. Tetrazolo-172 and Oxo-172 are thus potential candidates for further development.

Though the most active thiazolidinones contain a negative charge at neutral pH, compound Pyridine-NO-172, which has IC_{50} of 9 μM for CFTR inhibition, is zwitterionic and net neutral. Together with analysis of site-directed CFTR mutants,[13] the existence of a moderately potent CFTR inhibitor with net neutral charge indicates that a negative charge is not necessary for CFTR inhibition by thiazolidinones. Though Pyridine-NO-172 is not a candidate for further development because of its predicted poor in vivo stability, it may be possible to identify other net neutral thiazolidinones with improved accumulation in cytoplasm compared to $CFTR_{inh}$-172.

A recent study from our laboratory identified Tetrazolo-172 as the best thiazolidinone for inhibition of renal cyst growth in models of polycystic kidney disease.[9] Prevention of cyst formation by Tetrazolo-172 in an MDCK cell model was substantially better than

Table 2
Variations in Ring B and Linkers 1 and 2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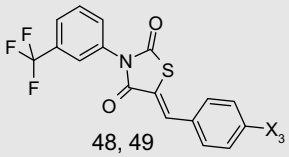
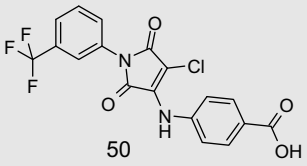
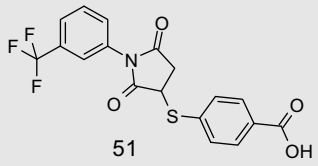

48, 49


50

Compound	X_3	IC_{50} (μM)
47		Inactive
48 Oxo-172	COOH	1.4
49	(2H)-Tetrazolo-5-yl	17
50		36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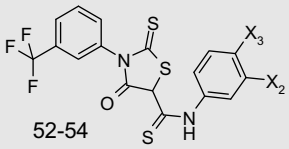
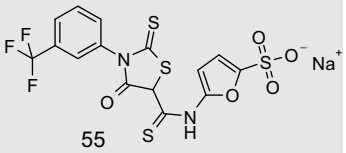
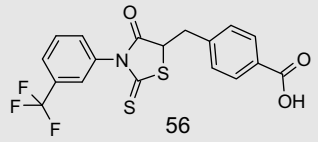

52-54


55

Compound	X_2	X_3	IC_{50} (μM)
51			7
52	H	COOH	9
53	OH	COOH	11
54	H	SO_3Na	25
55			27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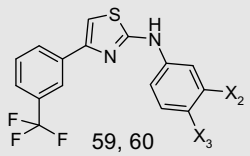
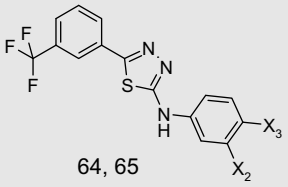
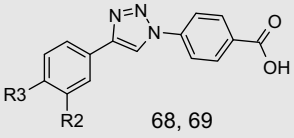

59, 60


64, 65


68, 69

Compound	X_3/R_3	X_2/R_2	IC_{50} (μM)
56			27
59	COOH	H	28
60	OH	COOH	32
64	COOH	H	Inactive
65	OH	COOH	Inactive
68	H	CF_3	Inactive
69	CF_3	H	Inactive

Table 3
Inhibition potency, solubility, and toxicity of CFTR inhibitors

Compound	IC_{50} (μM)	Solubility in saline (μM)	% Cell viability	
			20 μM	50 μM
$CFTR_{inh}$-172	0.38 ± 0.04	17	86	not soluble
Tetrazolo-172	0.76 ± 0.2	189	87	73
Oxo-172	1.4 ± 0.2	420	93	91
α-Me-172	8.2 ± 0.4	259	89	72
Pyridine-NO-172	8.7 ± 0.7	264	not determined	

by $CFTR_{inh}$-172. Tetrazolo-172 reduced cyst formation and expansion in an embryonic kidney organ culture model and in a mouse model of *pkd1* gene deletion. Whether Tetrazolo-172 or other small-molecule CFTR inhibitors are effective in human polycystic kidney disease will require clinical trials. Our recent study also identified a cell permeable phenyl derivative of the glycine hydrazide-type CFTR inhibitor as effectively reducing cyst formation and growth in in vitro and mouse models of polycystic kidney disease.

In contrast to polycystic kidney disease, which is a life-long condition, therapy of enterotoxin-mediated secretory diarrheas such as cholera or Traveler's diarrhea requires short-term therapy of days or less. Small-molecule CFTR inhibitors are predicted to reduce intestinal fluid secretion. Thiazolidinones, as 'absorbable-type' CFTR inhibitors that act from the cytoplasmic surface of CFTR, are taken up into enterocytes and enter the systemic circulation. Absorbable-type CFTR inhibitors are expected to resist potential

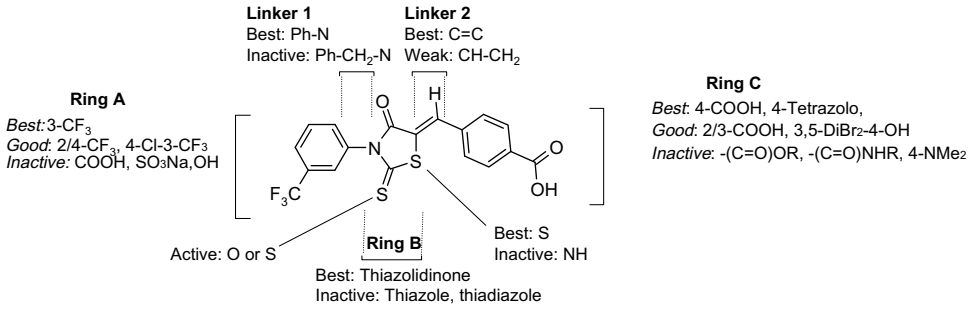


Figure 3. Structure–activity analysis of thiazolidinone CFTR inhibitors.

washout, a theoretical concern of non-absorbable CFTR inhibitors in which rapid fluid transit through the intestine may dilute and wash out compounds that weakly associate with targets on the surface of the intestinal lumen. Our laboratory has developed a series of non-absorbable CFTR inhibitors that block the CFTR pore from its external surface,[42,43] including macromolecular conjugates that stick tightly to the intestinal surface.[44] The clinical utility of absorbable vs. non-absorbable CFTR inhibitors in the treatment of secretory diarrheas will require human clinical trials.

4. Experimental methods

4.1. Synthesis procedures

^{1}H nuclear magnetic resonance spectra were obtained in $CDCl_3$ or dimethyl sulfoxide (DMSO)-d_6 using a 400-MHz Varian Spectrometer referenced to $CDCl_3$ or DMSO. Mass spectrometry was done on a Waters LC/MS system (Alliance HT 2790+ZQ, HPLC: Waters model 2690, Milford, MA); HRMS were recorded at mass spectrometry facility, University of California, Riverside. Flash chromatography was done using EM silica gel (230–400 mesh), and thin-layer chromatography was performed on Merk silica gel 60 F254 plates (Darmstadt, Germany).

Intermediates were confirmed by mass spectrometry and ^{1}H NMR. Full analytical data for representative and the main compounds, including their intermediates, are provided. Melting points are uncorrected. Weakly active and inactive compounds and their intermediates were characterized by mass spectral analysis (LC/MS). Purity was determined by TLC and HPLC. Compounds with purity >95% were used for CFTR inhibition testing.

4.2. Synthesis of thiazolidinone intermediate 3

Typical procedure for Route 1:[15] An equimolar amount of carbon disulfide was added dropwise to an ice-cold solution of aniline **1** and triethylamine in ethyl acetate over 30 min (Scheme 1). After stirring overnight, a yellow dithiocarbamate was isolated by filtration and reacted with an equimolar amount of aqueous bromoacetic acid solution ($NaHCO_3$, pH 8-9). After 2 h, the solution was acidified (HCl), refluxed, and the resultant precipitate crystallized from ethanol to yield thiazolidinone intermediate **3**. *Typical procedure for Route 2:* A solution of isothiocyanate **2** (5 mmol, in THF) was added dropwise to a stirred aqueous solution of thioglycolic acid (0.347 g, 3.7 mmol) and triethylamine (1.38 ml, 10 mmol). After 30 min at 0 °C, the reaction mixture was further stirred at room temperature for 3 h. The reaction mixture was acidified (HCl), refluxed, and resultant precipitate crystallized from ethanol to yield thiazolidinone intermediate **3**.

Isothiocyanates and isocyanates **2**, if not available commercially, were prepared by reaction of respective amino compounds **1** with phosgene or thiophosgene, following known procedures.[29]

4.2.1. 2-Thioxo-3-[3-(trifluoromethyl)phenyl]-4-thiazolidinone (intermediate 3; R1 = H, R2 = CF_3, R3 = H, *n* = 0, Y and Z = S)

Mp 177–178 °C; ^{1}H NMR ($CDCl_3$): δ 7.72 (d, 1H, phenyl, *J* = 7.6 Hz), 7.64 (t, 1H, phenyl, *J* = 8.0 Hz), 7.48 (s, 1H, phenyl), 7.40 (d, 1H, phenyl, *J* = 8.0 Hz), d 4.18 (s, 2H, CH2); MS (ES^+) (*m*/*z*): $[M+1]^+$ calculated for $C_{10}H_6F_3NOS_2$, 278.29, found 277.93.

4.2.2. 2-Thioxo-3-[2-methyl-3-(trifluoromethyl)phenyl]-4-thiazolidinone

MS (ES^+) (*m*/*z*): $[M+1]^+$ calculated for $C_{11}H_8F_3NOS_2$, 292.32, found 292.02.

4.3. Synthesis of compounds 5–41, 47, 48

4.3.1. 4-[[4-Oxo-2-thioxo-3-[3-(trifluoromethyl)phenyl]-5-thiazolidinylidene]methyl]benzoic acid (5, CFTRinh-172)[14]

A mixture of 2-thioxo-3-(3-trifluoromethyl phenyl)-4-thiazolidinone **3** (55 mg, 0.2 mmol,[15] 4-carboxybenzaldehyde (30 mg, 0.2 mmol), and a drop of piperidine in absolute ethanol (0.5 ml) was refluxed for 2 h. Solvent was evaporated, and the residue was crystallized from ethanol and further purified by normal phase flash chromatography to yield 54 mg yellow powder (yield 67%); mp 182–183 °C; ^{1}H NMR (DMSO-d_6): δ 13.20 (bs, 1H, COOH, D_2O exchange), 8.07 (d, 2H, carboxyphenyl, *J* = 8.31 Hz), 7.80–8.00 (m, 5H, trifluoromethyl-phenyl and CH), 7.78 (d, 2H, carboxyphenyl, *J* = 8.2 Hz); HRMS (ESI^-) (*m*/*z*): $[M-1]^-$ calculated for $C_{18}H_9F_3NO_3S_2$, 407.9976, found 407.9976.

4.3.2. 5-[[4-(2H-Tetrazol-5-yl)phenyl]methylene]-2-thioxo-3-[3-(trifluoromethyl)phenyl]-4-thiazolidinone (6, Tetrazolo-172)[9]

Mp 216–219 °C; ^{1}H NMR (DMSO-d_6): δ 11.92 (bs, 1H, tetrazolo-H, D_2O exchange), 8.16 (d, 2H, phenyl, *J* = 8.3 Hz), 8.02 (s, 1H), 7.92 (s, 1H), 7.87–7.84 (m, 3H, phenyl or trifluoromethyl-phenyl and/or CH), 7.79–7.76 (m, 2H, trifluoromethyl-phenyl); MS (ES^-) (*m*/*z*): $[M-1]^-$ calculated for $C_{18}H_9F_3N_5OS_2$, 432.0201, found 432.0205.

4.3.3. 5-(1-Oxido-4-pyridinyl)methylene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-4-thiazolidinone(17, Pyridine-NO-172)[13]

Mp 209–210 °C (decomp); MS (ES+) (*m*/*z*): $[M+H]^+$ calculated for $C_{16}H_9F_3N_2O_2S_2$, 382.39, found 383.01.

4.3.4. 4-[[4-Oxo-2-thioxo-3-[2-methyl-3-(trifluoromethyl)phenyl]-5-thiazolidinylidene]methyl] benzoic acid(18, α-Me-172)

Mp 156–158 °C; ^{1}H NMR (DMSO-d_6): δ 12.74 (bs, 1H, COOH, D_2O exchange), 8.05 (d, 2H, carboxyphenyl, *J* = 8.3 Hz), 7.90 (1H, s, =CH—), 7.85 (d, 1H, *J* = 7.8 Hz, trifluoromethylphenyl), 7.79 (d, 2H, *J* = 8.3, carboxyphenyl), 7.74 (d, 1H, *J* = 7.3 Hz, trifluoromethyl-phenyl), 7.58 (t, 1H, *J* = 7.8 Hz, trifluoromethylphenyl), 2.13 (s, 3H, CH_3); HRMS (ESI^-) (*m*/*z*): $[M-1]^-$ calculated for $C_{19}H_{11}F_3NO_3S_2$, 422.0132, found 422.0140.

4.3.5. 5-(4-Pyridinylmethylene)-2-thioxo-3-[3-(trifluoromethyl) phenyl]-4-thiazolidinone (33)

Mp 186–188 °C; ^{1}H NMR (DMSO-d_6): δ 8.72 (dd, 2H, J = 6.35, 2.93 Hz, pyridine), 7.92 (s, 1H, =CH-), 7.87 (d, 1H, J = 7.32 Hz, trifluoromethylphenyl), 7.81–7.75 (m, 3H, trifluoromethylphenyl), 7.59 (dd, 2H, J = 6.35, 2.93 Hz, pyridine); MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{16}H_9F_3N_2OS_2$, 367.40, found 367.20.

4.3.6. 4-[[4-Oxo-2-thioxo-3-[(3-(trifluoromethyl)phenyl) methyl]-5-thiazolidinylidene]methyl]benzoic acid (46)

^{1}H NMR (DMSO-d_6): δ 13.19 (s, 1H, COOH), 8.01 (d, 2H, J = 8.3 Hz, carboxyphenyl), 7.86 (s, 1H, trifluoromethylphenyl), 7.73–7.70 (m, 3H, carboxyphenyl and trifluoromethylphenyl), 7.63–7.51 (m, 3H, trifluoromethyl and =CH—), 5.29 (s, 2H, CH_2); MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{19}H_{12}F_3NO_3S_2$, 424.45, found 424.17.

4.3.7. 4-[[3-[3-(trifluoromethyl)phenyl]-2,4-dioxo-5-thiazolidinylidene]methyl]benzoic acid (48, Oxo-172)

Mp 168–170 °C; ^{1}H NMR (DMSO-d_6): δ 13.05 (bS, 1H, COOH, D_2O exchange), 8.06–8.04 (d, 2H, carboxyphenyl, J = 8.3 Hz), 8.01 (s, 1H), 7.92 (s, 1H), 7.86–7.84 (m, 1H), 7.78–7.74 (m, 4H); HRMS (ESI$^-$) (m/z): [M−1]$^-$ calculated for $C_{18}H_9F_3NO_4S$, 392.0204, found 392.0207.

4.3.8. 4-[[2,5-dioxo-1-[3-(trifluoromethyl)phenyl]-3-pyrrolidinyl] thio]-benzoic acid (51)

MS (ES$^-$) (m/z): [M−1]$^-$ calculated for $C_{18}H_{12}F_3NO_4S$, 394.35, found 394.18.

4.3.9. 4-Oxo-[(3-trifluoromethyl)phenyl]-2-thioxo-N-[4-(carboxy)phenyl]-5-thiazolidinethio carboxamide (52)

MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{18}H_{11}F_3N_2O_3S_3$, 457.49, found 457.37.

4.3.10. 4-Oxo-[(3-trifluoromethyl)phenyl]-2-thioxo-N-[(4-carboxy-3-hydroxy)phenyl]-5-thiazolidinethiocarboxamide (53)

MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{18}H_{11}F_3N_2O_4S_3$, 473.50, found 473.23.

4.3.11. 4-[[4-Oxo-2-thioxo-3-[3-(trifluoromethyl)phenyl]-5-thiazolidinyl]methyl]benzoic acid (56)[24,34]

To a stirred solution of **5** (102 mg, 0.25 mmole) in pyridine (1 ml), lithium borohydride (1 M solution in THF, 500 μl) was added dropwise over 30 min. The reaction mixture was refluxed (2 h), cooled, and added to an ice-cold solution of hydrochloric acid (4 N, 5 ml). The resulting material was refluxed for 30 min, filtered hot, and cooled. The precipitate was dried to yield **56**. MS (ES+) (m/z): [M−1]$^-$ calculated for $C_{18}H_{12}F_3NO_3S_2$, 410.42, found 410.13.

4.3.12. 3-Trifluoromethyl benzoic acid hydrazide

Hydrazine hydrate (4 equivalents) was added to the stirred solution of 3-trifluoromethyl benzoyl chloride **61** in pyridine at 0 °C. The reaction mixture was added to the ice-cold water and the collected precipitate was recrystallized from ethanol (yield 83%). MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_8H_7F_3N_2O$, 205.16, found 205.01.

4.3.13. 3-Trifluoromethyl-2-[[(4-carboxyphenyl) amino]thioxomethyl]benzoic acid hydrazide (62)

A mixture of 3-trifluoromethyl benzoic acid hydrazide (1 g, 5 mmol, prepared as above) and appropriate carboxyphenylisothiocyanate (5 mmol) in THF was refluxed for 2 h. The solid obtained after solvent evaporation was purified by recrystallization from ethanol to give thiosemicarbazide **62** (yield 74%). MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{16}H_{12}F_3N_3O_3S$, 384.35, found 384.06.

4.3.14. 4-[(5-(3-Trifluoromethylphenyl)-1,3,4-thiadiazol-2-yl) amino]-benzoic acid (64)[37]

Thiosemicarbazide **62** (2.6 mmol) was added slowly to 10 ml of concentrated sulfuric acid, stirred for 30 min at room temperature, and slowly dumped into a stirred ice-water mixture. The precipitated product was purified by flash chromatography to give **64** (yield 77%). ^{1}H NMR (DMSO-d_6): δ 10.98 (bs, 1H, COOH), 8.14–8.11 (m, 3H, trifluoromethylphenyl), 7.90 (d, 2H, J = 8.3 Hz, carboxyphenyl) 7.84–7.71 (m, 4H, (2H carboxyphenyl, 1H trifluoromethylphenyl, NH)); MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{16}H_{10}F_3N_3O_2S$, 366.35, found 366.46.

4.3.15. 4-Hydroxy-3-[[5-(3-trifluoromethyl)phenyl-1,3,4-thiadiazol-2-yl]amino]-benzoic acid (65)

Synthesized by same procedure as for **64**. MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{16}H_{10}F_3N_3O_2S$, 382.34, found 382.03.

4.3.16. 4-[4-[3-(trifluoromethyl)phenyl]-1H-1,2,3-triazol-1-yl]-benzoic acid (68)[38]

1-Ethynyl-3-(trifluoromethyl)benzene (0.85 g, 5 mmol) and 4-azidobenzoic acid (0.815 g, 5 mmol) were suspended in a 1:1 mixture of water and *tert*-butyl alcohol (10 mL). A freshly prepared solution of sodium ascorbate (0.3 mmol, 300 μL of 1 M) was added, followed by copper (II) sulfate pentahydrate (7.5 mg, 0.03 mmol, in 100 μL of water). The mixture was stirred for 24 h at room temperature. The reaction mixture was diluted with water, and the white precipitate was collected by filtration, washed, and dried to give 1.53 g (92%) of pure product as an off-white powder. Mp >250 °C; MS (ES$^+$) (m/z): [M+1]$^+$ calculated for $C_{16}H_{10}F_3N_3O_2$, 334.28, found 334.09.

4.3.17. Solubility

As reported,[45] a saturated compound solution was prepared by addition of DMSO stock to phosphate buffered saline (final DMSO 2%) followed by sonication for 5 min at 25 °C and shaking at room temperature for 1 h. After centrifugation at 15,000 rpm for 1 h, the supernatant was analyzed by LC/MS with concentration determined from area under the curve, standardized against calibration data.

Standard curves for each compound were obtained by plotting area under the curve from chromatograms against inhibitor concentration. The concentration range of the standard solutions was 1–15 μM (for **5**) and 1–100 μM (other compounds). In all cases, standard curves prepared were linear with r > 0.998.

4.3.18. Short-circuit current measurements

FRT cells (stably expressing human wildtype CFTR) were cultured on Snapwell filters with 1 cm^2 surface area (Corning-Costar) to resistance >1000 Ω cm^2 as described.[11] Filters were mounted in an Easymount Chamber System (Physiologic Instruments, San Diego). For apical Cl^- current measurements the basolateral hemichamber contained (in mM): 130 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 Na–HEPES, 10 glucose (pH 7.3). The basolateral membrane was permeabilized with amphotericin B (250 μg/ml) for 30 min. In the apical solution 65 mM NaCl was replaced by sodium gluconate, and $CaCl_2$ was increased to 2 mM. Solutions were bubbled with 95% O_2/5% CO_2 and maintained at 37 °C. Current was recorded using a DVC-1000 voltage-clamp (World Precision Instruments) using Ag/AgCl electrodes and 1 M KCl agar bridges.

4.3.19. Cytotoxicity

FRT cells in confluent monolayers were incubated with compounds for 2 days. Cells were washed 3 times, fixed (cytofix, 30 min), and stained with crystal violet (100 μl, 0.5%, 10 min) using standard procedures.[46] Excess crystal violet was removed by washing and dye was extracted with Sorenson's buffer (0.1 M sodium

citrate, 50% ethanol, pH 4.2). Crystal violet was quantified by measurement of absorbance at 650 nm. Percentage crystal violet staining was determined from test wells measured 8 times, compared to blanks (wells not containing cells) and vehicle-treated cells.

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