High-throughput Surface Liquid Absorption and Secretion Assays to Identify F508del CFTR Correctors Using Patient Primary Airway Epithelial Cultures

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Supplemental Material

Materials and Methods

Patient Primary Airway Cells

F508del/F508del patient bronchial epithelial cells at passage 1 were obtained from Tissue Procurement & Cell Culture at the Cystic Fibrosis Center of the University of North Carolina Chapel Hill. The research conducted on human cells at UNC-CH has been verified as compliant with Pfizer policies including institutional review board or institutional ethics committee approval. Cells from patient code R were used for all of the screening with surface liquid assays reported herein. Corrector activity of compounds 1, 4, 7, 8, 9, 10, and 12 was confirmed by an orthogonal electrophysiology assay in at least one patient code different from that used in the primary screen as described in Table S6. Cells from patient code Y were used for the Western blot in Figure 5C.

CF hBE Equivalent Current Assay

Fully-differentiated CF hBE cultures in Transwell-24 filter plates (cat. no. 3378, Corning Inc., Corning, NY), were treated with test compound at various concentrations for 40 - 46 hours on the basolateral side. VX-809 at 1 - 5 µM was used as a positive control and 0.2% (v/v) DMSO vehicle as negative control.

Compounds and medium were removed from the cells and replaced with apical and basolateral assay buffer (Nutrient Mixture Ham's F12 with Coon's Modification (Sigma-Aldrich, St. Louis, MO) plus 20 mM HEPES and 11 mM Tris, pH 7.4). After equilibration at 36^{o}C, voltage and resistance were measured using a 24-channel transepithelial current clamp apparatus (Transepithelial Current Clamp TECC24, EP Design, Bertem, Belgium). Amiloride (30 µM, Sigma-Aldrich) was added to the apical side to inhibit ENaC activity. Forskolin (10 µM, Tocris, Bristol, UK) and VX-770 (0.1 µM) were then added simultaneously to fully activate CFTR. Lastly, chloride transport was blocked by the addition of the Na-K-Cl co-transporter antagonist bumetanide (30 µM, Sigma-Aldrich).

CFTR current (expressed as µA/cm^2) was calculated from the measured voltage and resistance then integrated over time for the period of forskolin plus VX-770 stimulation to generate an area under the curve (AUC). The AUC was divided by the time period of integration and reported as the area under the curve per minute (µA/cm^2/min). Test compound activity was scaled relative to activity in cells treated with VX-809 after subtraction of the DMSO vehicle response.

CFTR Western Blot in HEK Cell Line

HEK293 cells were washed, harvested in ice-cold PBS, then centrifuged at 500 x g for 5 minutes at 5^{o}C. Cell pellets were lysed with Tris-HCl, pH 7.4, 1% v/v Igepal (Sigma-Aldrich), 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich), 150 mM NaCl, 5 mM sodium EDTA) containing protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich), vortexed, then incubated for 30 minutes on ice. Lysates were cleared by centrifugation at 14,000 x g for 10 minutes at 4^{o}C. Protein concentration was determined by Pierce 660 nm Protein Assay (ThermoFisher Scientific, Waltham, MA). Samples were prepared in 1X NuPAGE LDS Sample Buffer (ThermoFisher Scientific) containing 5% beta-mercaptoethanol (Sigma-Adrich), heated for 10 minutes at 37^{o}C and separated on NuPAGE 3-8% Tris-Acetate protein gels for 2 hours at 100 V. Proteins were transferred onto nitrocellulose membranes using the Turbo Transfer system (Bio-Rad, Hercules, CA) and the membranes blocked in Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NE), overnight at 4^{o}C. All primary and secondary antibody incubations were in blocking buffer + 0.1% Tween-20 for either 2 hours (primary) or 1 hour (secondary) at room temperature. CFTR was detected using monoclonal antibody UNC 596 (CFFT Antibody Distribution Program, www.cff.org) at a 1:3000 dilution. Loading control antibodies for alpha- or beta-tubulin (Cell Signaling Technologies, Danvers, MA) were used at dilutions of 1:5000. Membranes were washed 4 times with TBS-T for 10 minutes each before probing with IRDye 800CW anti-mouse IgG (H + L) or IRDye 800CW ant-rabbit (H +L) IgG secondary antibody conjugates (LI-COR Biotechnology) for 30 minutes at room temperature. Membranes

were washed 4 times with PBS-T for 10 minutes followed by one wash with PBS and detection on a C-DiGit blot scanner (LI-COR Biotechnology).

CFTR Western Blot in Primary Airway Epithelia

CF hBE cells on filters were washed twice with ice-cold PBS then lysed by scraping with a pipette tip in 20 mM Tris HCl, pH 7.5 1% NP-40 substitute, 0.5% deoxycholate, 200 mM sodium chloride, 1 mM EDTA with added protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich). The lysate was incubated on ice for 1 hour with mixing every 15 min, then centrifuged at 14,000 rpm for 10 minutes at 4^{o}C. Protein concentration, sample buffer, electrophoresis, transfer, blocking, and primary antibody conditions were as described above. CFTR was detected using monoclonal antibody UNC 596 (CFFT Antibody Distribution Program) at 1:2500 dilution. Loading control antibody for beta-tubulin (Cell Signaling Technologies) was used at a dilution of 1:1000. Membranes were washed 6 times with TBS-T for 5 minutes each before probing with either donkey anti-mouse (H + L) IgG or donkey anti-rabbit (H +L) IgG secondary antibody-HRP conjugates (Jackson ImmunoResearch West Grove, PA; 1:10,000 dilution) for 30 minutes at room temperature. Membranes were washed with TBS-T six times for 5 minutes and then once for 10 minutes. Signal from SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher) was acquired on an ImageQuant LAS 4000 scanner (GE Healthcare Life Sciences, Pittsburgh, PA).

Immunofluorescence

Cells on Transwell filters were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, blocked with 1% BSA/3% normal goat serum in PBS containing 0.1%

Triton X-100 for 1 hour at room temperature, then incubated with a mixture of mouse anti-acetylated tubulin monoclonal antibody (1:500 dilution, cat. no. T7451, Sigma-Aldrich) and rabbit anti-mucin 5B polyclonal antibody (1:500 dilution, cat. no. sc-20119, Santa Cruz Biotechnology, Dallas, TX) in PBS containing 1% BSA/0.1% Triton X-100 overnight at 4^{o}C. After washing 3 times for 5 min with PBS, cells were incubated with a solution of Alexa Fluor 488 goat anti–mouse IgG antibody (2 µg/mL, cat. no. A32723, ThermoFisher Scientific) and Alexa Fluor 594 goat anti–rabbit (2 µg/mL, cat. no. R37117, ThermoFisher Scientific) for 2 hours, washed with PBS, incubated with biotinylated peanut agglutinin (2 ug/mL, cat. no. BA-0074, Vector Laboratories, Burlingame, CA) for 1 hour, washed 3 times with PBS followed by 1 hour incubation with Alexa Fluor 647 streptavidin (1:1000 dilution, cat. no. S21374, ThermoFisher Scientific), and washed with PBS. Membrane filters were mounted face up using ProLong Gold Antifade Mountant with DAPI (cat. no. P36935, ThermoFisher Scientific). Fluorescence images were acquired with a 20X objective (200X magnification) using an Axio Imager microscope platform (Carl Zeiss Microscopy, Thornwood, NY).

HEK Cell Line Ion Flux Assay

The F508del M470 cDNA was synthesized by System Biosciences (Palo Alto, CA). The sequence contained the following differences from the canonical CFTR cDNA sequence (NCBI Accession Number NM_000492): del CTT (1521-1523) to make F508del; synonymous changes T>C (798), A>G (801), T>C (804) to remove a cryptic bacterial promoter; synonymous change T>C (1095); G>A (1408) to give M470. The cDNA was amplified using primers 5’ gttgctagcggtaccatgcagaggtcgcctctgga-3’ and 5’

gttgcggccgcctaaagccttgtatcttgcacctc 3' and inserted into pcDNA5/FRT (Flp-In™, ThermoFisher Scientific) using Kpn1 and Not1 restriction sites.

HEK293 Flp-In cells (ThermoFisher Scientific) were cultured as recommended by the supplier. The CFTR cDNA was inserted according to the supplier's instructions using the mammalian expression vector and Flp recombinase vector pOG44.

Cells were seeded at 30,000 cells/well in 50 µL/well of growth medium in black-walled, clear-bottom, poly-D-Lysine-treated 384-well plates and incubated overnight at 37°C/5% CO_2. The next day medium was removed and replaced with 25 µL/well test compounds diluted in medium (final DMSO concentration 0.25%) before returning to the 37°C/5% CO_2 incubator overnight. The next day medium was removed and replaced with 25 µL/well of PBS containing 20 µM forskolin and 1 µM VX-770. After incubation at room temperature for 30 minutes, plates were loaded into a FLIPR Tetra fluorescence imaging plate reader (Molecular Devices, San Jose, CA) where 25 µL/well of iodide buffer (137 mM NaI, 1.5 mM K_2HPO_4, 8.1 mM NaH_2PO_4, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) was rapidly added with simultaneous measurement of fluorescence response over 20 seconds. The extent of fluorescence decrease at 20 seconds was used as a measure of CFTR activity. Activity was scaled relative to DMSO negative control (0%) and 5 µM corrector 4a (Tocris, Bristol, UK) positive control (100%).

<u>CFBE41o- Cell Surface Expression Assay</u>

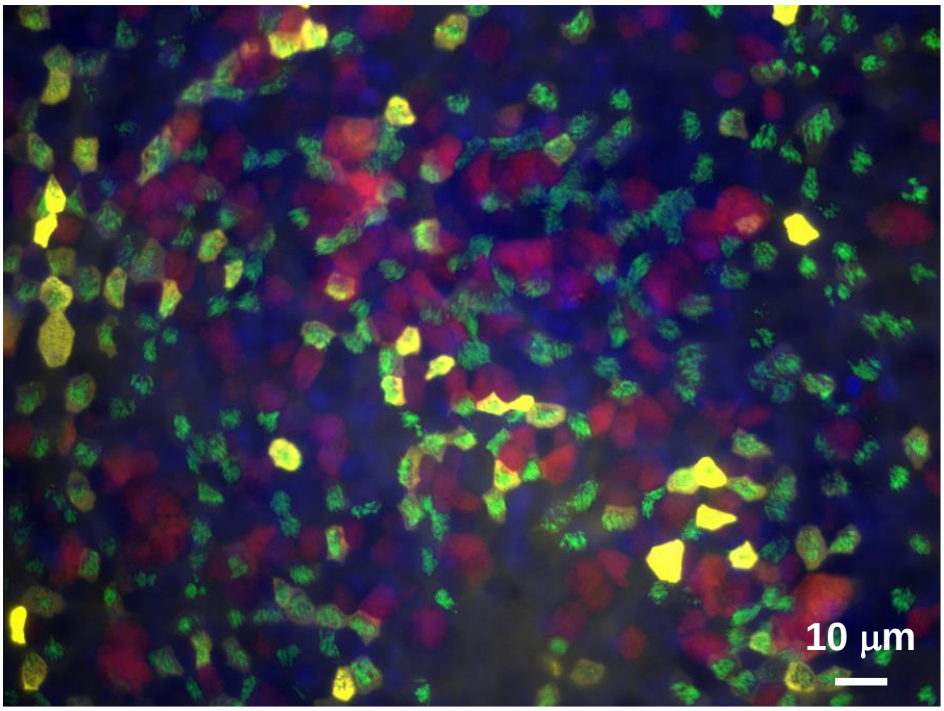
CFBE41o- cell lines stably expressing F508del R1S or F508del R1070W CFTR-3HA variants under the control of a tetracycline-responsive promoter were generated in the lab of Prof. Gergely Lukacs (McGill University, Montreal, Canada).[1] The phenotypic

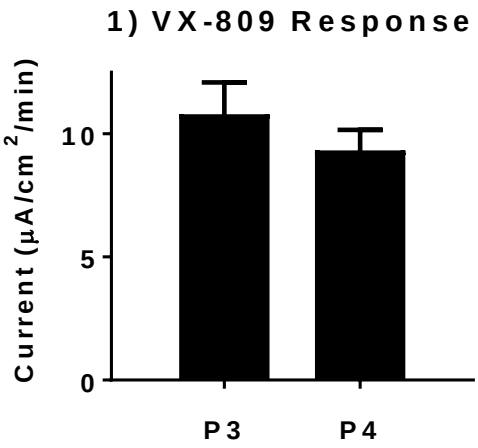
screen compound collection was screened for correctors in the lab of Dr. Martin Mense (Cystic Fibrosis Foundation Therapeutics Laboratory, Lexington, MA). Hits were tested for correction activity in CF hBE cells using an equivalent current electrophysiology assay at Pfizer.

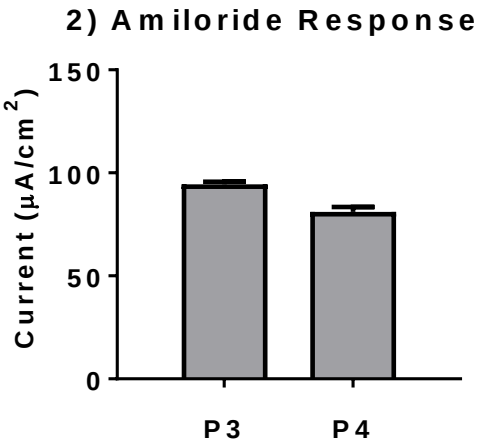
Results

Patient Primary Bronchial Epithelial Cells Maintain Their Ability to Differentiate and Respond to Correctors Up To Passage 4

We confirmed that F508del CF hBE cells maintained their capacity to differentiate into an epithelium at an air/liquid interface up to passage 4 using markers for specific cell types and functional measurements of epithelial ion channel activities. Passage 4 cells expressed acetylated tubulin (ciliated cells), peanut agglutinin (basal cells), and mucin 5B (goblet cells) (Figure S1A). In equivalent current electrophysiology assays, passage 3 and passage 4 cells gave similar amiloride responses (a measure of ENaC epithelial sodium channel activity) and similar responses to the clinical corrector VX-809 (a measure of rescue of the F508del mutation in CFTR) (Figure S1B). Passage 4 cells continuously cultured from passage 1 also gave similar amiloride and VX-809 responses to passage 4 cells made from a frozen passage 2 bank (Figure S1B). Passage 4 cells have amiloride responses and response to corrector VX-809 comparable to passage 3 cells. Cells can be cryopreserved at passage 2 and used at passage 4 with no loss of amiloride or VX-809 response.


10 μm


1) VX-809 Response
Current (μA/cm 2/min)
10
5
0
P3
P4


2) Amiloride Response
Current (μA/cm 2)
150
100
50
0
P3
P4

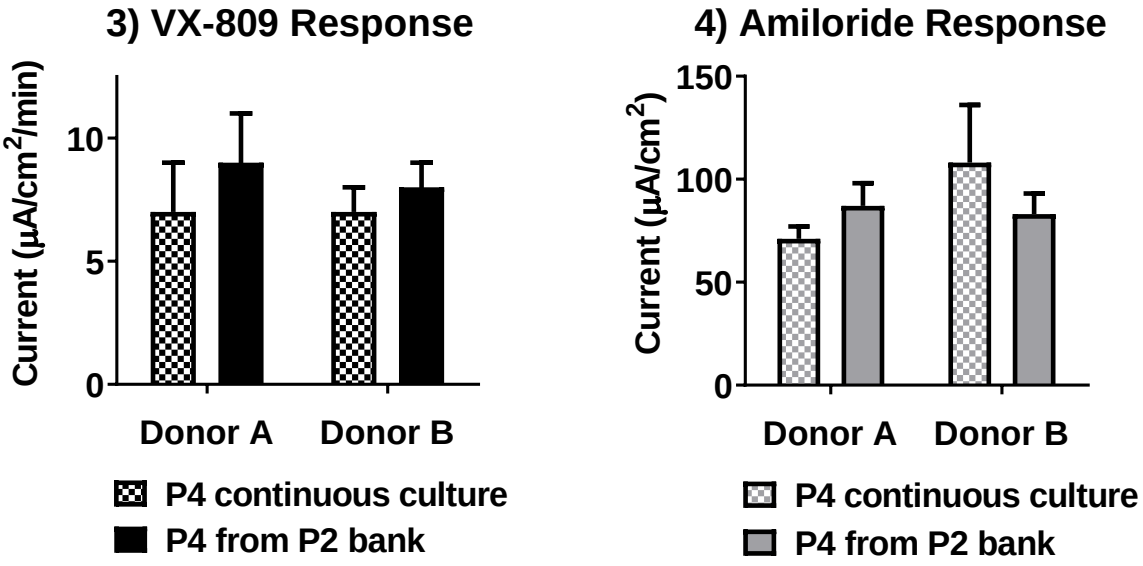


Figure S1. Upper) Immunofluorescence microscopy image of CF bronchial epithelial cells cultured on 96-well filter plates and stained for acetylated tubulin (green), mucin 5B (red), peanut agglutinin (yellow), and DAPI (blue). The scale bar at lower right indicates 10 µm. Lower) Equivalent current electrophysiology data in CF hBE cells (mean response and standard deviation). 1) Comparison of VX-809 responses for passage 3 and passage 4 cells. 2) Comparison of amiloride responses for passage 3 and passage 4 cells. 3) Comparison of VX-809 responses for passage 4 cells cultured continuously from passage 1 thaw and passage 4 cells cultured from a frozen passage 2 bank. 4) Comparison of amiloride responses for passage 4 cells cultured continuously from passage 1 thaw and passage 4 cells cultured from a frozen passage 2 bank.

Image Analysis to Measure Meniscus Width

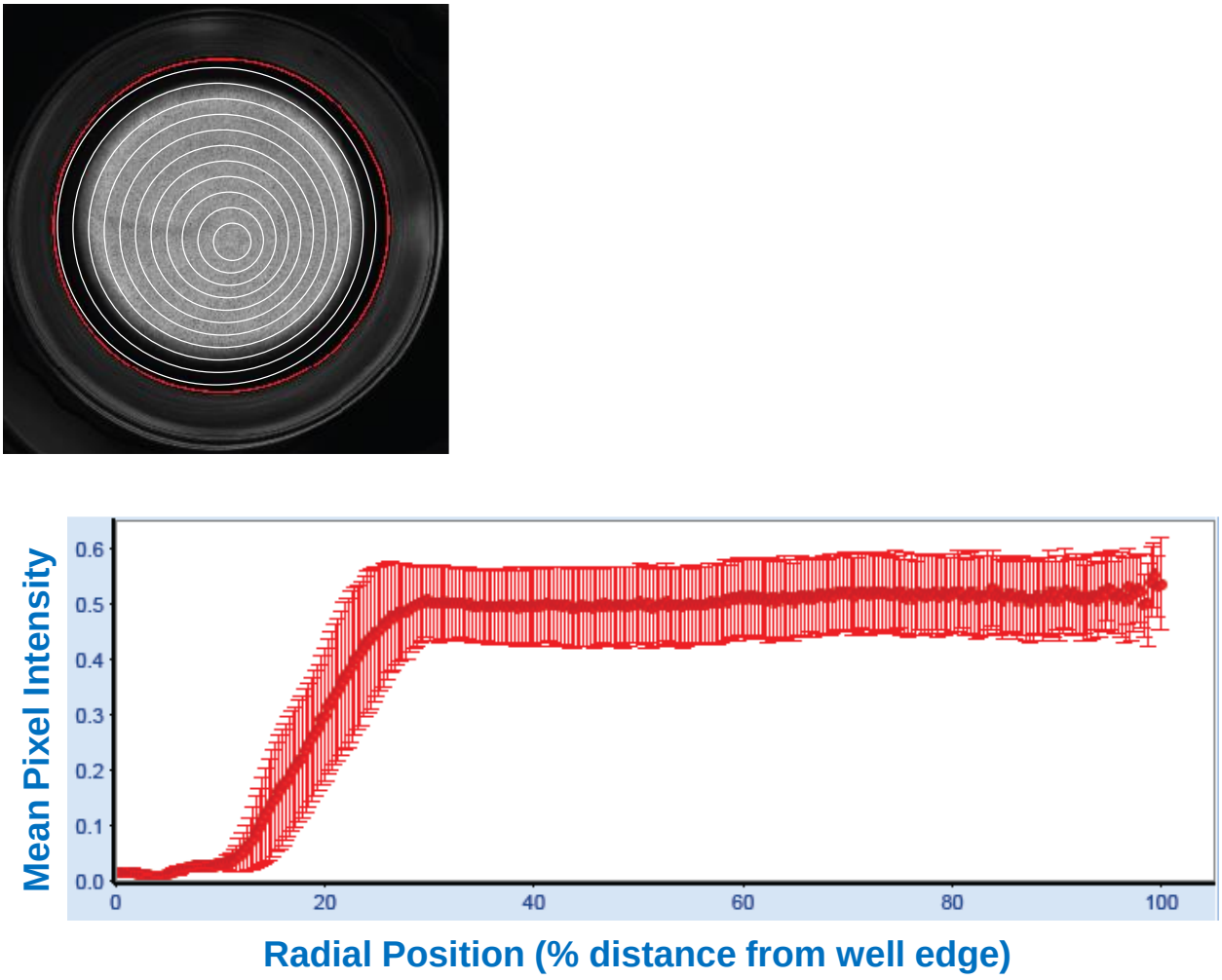


Figure S2. Upper) Brightfield images acquired at low magnification (2X) were processed to measure meniscus width using a custom algorithm in Pipeline Pilot software (BIOVIA, San Diego, CA). A ring was drawn around the well edge (red). Pixel intensity was measured in contracting concentric rings from the edge to the center of each well (white). Lower) Mean pixel intensity was plotted against the distance from the well edge and the position of the half-maximal pixel intensity calculated using a 4-parameter logistical fit.

Configuration of a Surface Liquid Absorption Assay to Screen Multiple Compounds per Well

We used a set of compounds randomly selected from a screening collection to prepare DMSO stocks that resulted in 6, 9, or 12 compounds per well, each at 5 or 10 µM final concentration. These compound mixtures were overlaid onto either DMSO or VX-809, maintaining a final DMSO concentration of 0.2%. With 9 compounds each at 10 µM and 12 compounds each at 5 µM, the increased compound burden substantially reduced the VX-809 response (data not shown). Evaluation of the 6 compounds each at 10 µM showed that the increased compound burden did not significantly affect the average DMSO and VX-809 responses. While some compound mixtures did suppress the VX-809 response, the reduction was generally less than 25% (Figure S2).

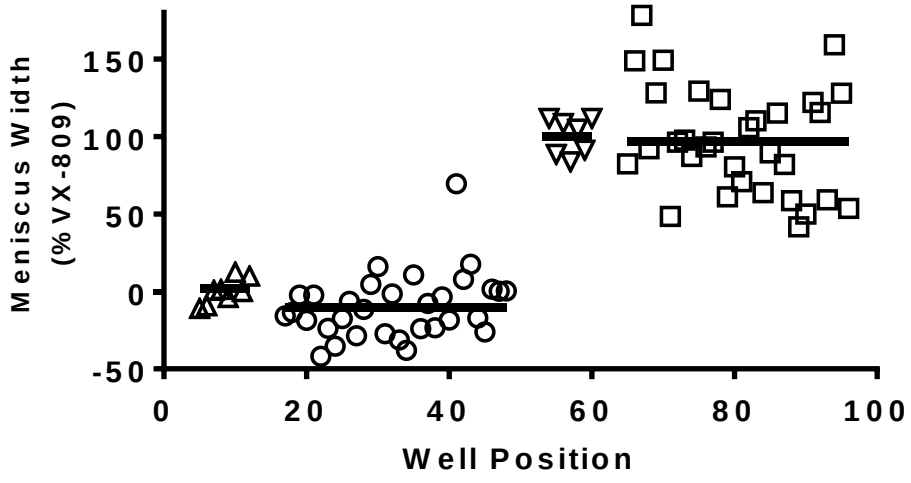


Figure S3. Meniscus width data for wells treated with DMSO (triangles), VX-809 (inverted triangles), mixtures of 6 compounds plus DMSO (open circles), or mixtures of 6 compounds plus VX-809 (open squares) and their average responses (thick horizontal lines).

Pilot Screen Using The Absorption Assay

Table S1. Summary of the outcomes of the liquid absorption screen of the 8414 compound phenotypic screen library, hit deconvolution, and hit confirmation.

Compounds screened (6 compounds per well)	8414
Mixtures of 6 screened	1402
Active mixtures of 6 (>10% of VX-809 response)	173
Single compounds tested for deconvolution	1023
Confirmed ASL active single compounds (>10% of VX-809 response)	90
Single compounds with activity in equivalent current electrophysiology assay >150% of VX-809 response when combined with VX-809	6

Table S2. Activities of confirmed hits from screening of the phenotypic screen library using the CF hBE cell liquid absorption assay in patient code R. ND = not determined. Average ± SEM.

Compound No.	**Liquid Absorption Assay**		**Equivalent Current Assay**			
			Compound Alone		**Compound Plus VX-809**	
	(μM)	(VX-809 = 100)	(μM)	(VX-809 = 100)	(μM)	(VX-809 = 100)
	Conc.	Activity	Conc.	Activity	Conc.	Activity
1	8	38 ± 6	10	103 ± 11	10	348 ± 29
2	8	36 ± 3	ND	ND	10	170 ± 2
3	8	29 ± 5	10	55 ± 9	10	189 ± 24
4	8	26 ± 3	10	29 ± 4	10	248 ± 14
5	8	21 ± 7	10	40 ± 1	10	158 ± 17
6	8	19 ± 3	10	18 ± 7	10	150 ± 9

Pilot Screen Using The Liquid Secretion Combination Assay

The secretion combination assay based on compound A was also used to screen the phenotypic screen library. Six confirmed hits were identified, including compound 1 which was identified by the absorption assay. All of the other compounds were different from the confirmed hits from the absorption assay format in the absence of compound A. None of these other compounds when combined with VX-809 increased the response to >150% of VX-809 alone in the CF hBE equivalent current electrophysiology assay (data not shown). We concluded that the inclusion of compound A likely sensitized the assay to different F508del correction mechanisms and apparently also increased its stringency. The increased stringency was in part because compound A was used at its maximally-efficacious concentration, so that compounds having a similar mechanism would be unlikely to further increase ASL secretion and would not be detected.

Table S3. Activities of confirmed hits from screening of the phenotypic screen library using HEK cell lines expressing F508del together with R1070W or R555K suppressor mutations. The compound collection was screened at both 1 and 10 µM in both mutants. ND = not determined. Average ± SEM.

Compound No.	HEK F508del Ion Flux Assay			Equivalent Current Assay			
		R1070W	R555K	Compound Alone		Compound + VX-809	
	(µM)	(VX-809 = 100)		(µM)	(VX-809 = 100)	(µM)	(VX-809 = 100)
	Conc.	Activity		Conc.	Activity	Conc.	Activity
13	10	66 ± 4	42 ± 0	10	-22 ± 1	8	73 ± 24
1	10	65 ± 2	39 ± 1	10	103 ± 11	10	348 ± 29
14	10	44 ± 1	23 ± 0	10	-10 ± 3	10	92 ± 16
15	10	41 ± 1	11 ± 4	3	18 ± 2	10	203 ± 22
16	10	38 ± 4	14 ± 4	10	ND	10	100 ± 10
17	10	38 ± 2	10 ± 1	10	-16 ± 3	10	76 ± 6
18	10	36 ± 13	24 ± 3	10	23 ± 12	10	128 ± 16
19	10	27 ± 3	20 ± 1	10	-5 ± 2	10	116 ± 9
20	10	26 ± 1	21 ± 1	10	-3 ± 3	10	104 ± 2
21	10	15 ± 4	50 ± 45	10	-1 ± 5	10	107 ± 12
22	10	9 ± 1	48 ± 41	10	-7 ± 4	10	118 ± 1
23	10	24 ± 2	37 ± 17	10	-12 ± 2	1	115 ± 4

Table S4. Activities of confirmed hits from screening of the phenotypic screen library using CFBE41o- cell lines expressing F508del together with R1070W or 3S suppressor mutations. The compound collection was screened at both 1 and 10 µM in both mutants. Average ± SEM.

Compound No.	CFBE41o- F508del Ion Flux			Equivalent Current Assay			
		3S	R1070W	Compound Alone		Compound + VX-809	
	(µM)	(VX-809 = 100)		(µM)	(VX-809 = 100)	(µM)	(VX-809 = 100)
	Conc.	Activity		Conc.	Activity	Conc.	Activity
24	10	15 ± 1	57 ± 8	1	-3 ± 2	1	99 ± 6
25	10	11 ± 1	42 ± 7	10	4 ± 6	10	100 ± 2
26	10	7 ± 1	33 ± 18 at 1 uM	4	9 ± 13	1	114 ± 2
27	10	15 ± 3	24 ± 2	10	-7 ± 2	10	92 ± 11
28	10	20 ± 0	18 ± 4	10	28 ± 3	10	137 ± 11
29	10	7 ± 1	24 ± 2	10	20 ± 1	10	128 ± 3
30	10	15 ± 1	23 ± 5	10	16 ± 2	10	83 ± 13

Table S5. Activities of confirmed hits from screening of 61,046 compounds from a general screening collection in the secretion combination assay in patient code R. ND = not determined. Average ± SEM.

Compound No.	Secretion Combination Assay		Equivalent Current Assay			
			Compound Alone		Compound + VX-809	
	(µM)	(VX-809 = 100)	(µM)	(VX-809 = 100)	(µM)	(VX-809 = 100)
	Conc.	Activity	Conc.	Activity	Conc.	Activity
7	8	100 ± 2	20	121 ± 5	10	303 ± 24
8	8	68 ± 10	10	46 ± 6	30	206 ± 10
9	8	49 ± 10	30	154 ± 5	30	296 ± 8
10	8	34 ± 9	ND	ND	30	200 ± 5
11	8	26 ± 4	5	9 ± 18	5	189 ± 4
12	8	20 ± 3	30	35 ± 4	30	205 ± 31

Table S6. Patient cells codes used for screening and confirmatory electrophysiology assays of selected hits from the liquid secretion and secretion combination screens. All patient codes tested showed responses to the corrector compounds tested. ND = not determined.

Compound No.	Patient Codes			
			Equivalent Current Assay	
	Absorption Assay	Secretion Combination Assay	Compound Alone	Compound + VX-809
1	R		R, T, U, X, Z	R, V, X, Y, Z
2	R		R	ND
3	R		R, S	R, Y
4	R		R, Y	R
5	R		R, U	Y
6	R		R	R
7		R	R, Y	R, W, Y
8		R	R, W, Y	R, W, Y
9		R	R, W, Y	R, W, Y
10		R	ND	W
11		R	R, Y	R, Y
12		R	R, Y	R, Y

Table S7. Liquid secretion compound A combination and equivalent current VX-809 combination dose response data for compounds 1, 7, and 9. Where possible the data were fitted to a 4-parameter logistical equation (the parameters were EC_{50}, Hill slope, and top; the bottom was fixed at 0 (secretion combination) or 100 (equivalent current combination) and EC_{50} constrained to values > 0). The number of data points fitted and the outputs of the fits are listed here.

Cmpd No.	Curve Fit Information				
	Secretion Compound A Combination Assay				
	No. Data Pts.	EC_{50} (μM) (95% CI)	Hill Slope (95% CI)	Top (95% CI)	R Square
1	22	No Fit			
7	85	8 (4 - 81)	0.9 (0.6 - 1.4)	150 (113 - 341)	0.82
9	723	14 (12 - 19)	1.3 (1.2 - 1.5)	137 (123 - 160)	0.91

Cmpd No.	Curve Fit Information				
	Equivalent Current VX-809 Combination Assay				
	No. Data Pts.	EC_{50} (μM) (95% CI)	Hill Slope (95% CI)	Top (95% CI)	R Square
1	103	3.2 (2.6 - 4.5)	2.3 (1.3 - 5.0)	322 (291 - 375)	0.67
7	197	1.4 (0.8 - 5.5)	0.8 (0.5 - 1.5)	274 (253 - 340)	0.52
9	156	6 (3 - 66)	0.8 (0.4 - 1.2)	340 (295 - 553)	0.54

References

1. Okiyoneda, T.; Veit, G.; Dekkers, J. F.; et al. Mechanism-based corrector combination restores DeltaF508-CFTR folding and function. *Nature chemical biology* **2013,** *9*, 444-54.