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Biological characterization of F508delCFTR protein processing by the CFTR Corrector ABBV-2222/GLPG2222

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Abbreviations

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; Fsk, forskolin; MTT, 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide; NBD, nucleotide binding domain; PBS, phosphate-buffered saline; RT, room temperature; Ivacaftor, N-(2,4-di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide; Lumacaftor, 3-(6-(1-(2,2-difluorobenzo[d][1,3]dioxol-5-yl)cyclopropanecarboxamido)-3-methylpyridin-2-yl)benzoic acid; Tezacaftor, 1-(2,2-difluoro-1,3-benzodioxol-5-yl)-N-[1-[(2R)-2,3-dihydroxypropyl]-6-fluoro-2-(1-hydroxy-2-methylpropan-2-yl)indol-5-yl]cyclopropane-1-carboxamide; WT, wild-type.

Abstract

Cystic Fibrosis (CF) is the most common monogenic autosomal recessive disease in Caucasians caused by pathogenic mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. Significant small molecule therapeutic advances over the past two decades have been made to target the defective CFTR protein and enhance its function. To address the most prevalent defect of the defective CFTR protein (i.e. F508del mutation) in CF, two biomolecular activities are required, namely correctors to increase the "amount" of properly folded F508delCFTR levels at the cell surface, and potentiators to allow the effective opening, *i.e.* "function" of the F508delCFTR channel. Combined, these activities enhance chloride ion transport yielding improved hydration of the lung surface and subsequent restoration of mucociliary clearance. To enhance clinical benefits to CF patients, a complementary "triple combination" therapy consisting of two corrector molecules, type 1 (C1) and type 2 (C2) with additive mechanisms along with a potentiator (P) are being investigated in the clinic for maximum restoration of mutated CFTR function (Hongyu et al., 2017). We report the identification and *in vitro* biological characterization of ABBV-2222/GLPG2222, a novel, potent and orally bioavailable C1 corrector developed by Abbvie-Galapagos, and currently in clinical trials that exhibits substantial improvements over the existing C1 correctors. This includes improvements in potency and drug-drug-interaction (DDI) compared to VX-809 (herein reported as Lumacaftor) and improvements in potency and efficacy compared to VX-661 (herein reported as Tezacaftor). ABBV-2222/GLPG2222 exhibits potent *in vitro* functional activity in primary patient cells harboring F508del/F508del CFTR with an EC_{50} <10 nM.

Significance Statement

To address the most prevalent defect of the defective CFTR protein (*i.e.* F508del mutation) in Cystic Fibrosis, Abbvie-Galapagos has developed ABBV-2222/GLPG2222, a novel, potent and orally bioavailable C1 corrector of this protein. ABBV-2222/GLPG2222 exhibits potent *in vitro* functional activity in primary patient cells harboring F508del/F508del CFTR and is currently in clinical trials that exhibits substantial improvements over the existing C1 correctors.

Introduction

Cystic Fibrosis is one of the most common lethal autosomal recessive Mendelian disorders (OMIM(TM)), the gene for which was discovered >25 years ago (Kerem et al., 1989; Rommens et al., 1989). Affecting an estimated 75,000 people worldwide with ~30,000 living in the United States (CFF), CF is caused by loss-of-function mutations in the *cftr* gene protein product; CFTR (Riordan et al., 1989) that belongs to the ATP-binding cassette (ABC) transporter family. CFTR is primarily expressed at the apical membrane of secretory epithelia of the airways, intestine, hepatobiliary and reproductive tracts, as well as the pancreas and sweat gland secretory coil, where it acts as a cAMP-regulated (Kartner et al., 1991) and ATP-gated anion channel (Hwang and Sheppard, 2009), (Hwang et al., 2018). Through its anion channel function, CFTR plays an important role in the transport of salt and water across these secretory epithelia, that is required for effective hydration of epithelial surfaces. Over 2000 CFTR genetic variants have been identified within the general and disease populations, and with systematic efforts, 336 disease-causing variants in addition to 35 variants with varying clinical consequence have been distinguished from neutral variants (Cutting, 2015). The most prevalent mutation present on at least one allele in ~ 90% of CF patients eliminates three base pairs, deleting phenylalanine 508 in the CFTR protein (*Phe508del; F508del*). To effectively treat patients carrying the most prevalent CF-causing mutation, there is a need for two biomolecular activities, namely CFTR correctors that will increase the amount of F508del-CFTR protein at the plasma membrane, and CFTR potentiators that will allow effective functioning (gating) of the F508del-CFTR protein (Cheng et al., 1990; Dalemans et al., 1991; Lukacs et al., 1993; Cai et al., 2011; Lukacs and Verkman, 2012). For CF patients with the homozygous F508del mutation, both preclinical and clinical data indicate that a combination of these biomolecular activities are required for restoring F508del-CFTR function to levels projected to yield a significant clinical benefit. The advent of novel CFTR modulator therapies targeting this underlying CFTR defect have resulted in the development and marketing of

potentiator, Kalydeco (Ivacaftor) (Van Goor et al., 2009); (Kalydeco) for patients with 33 CFTR mutations causing gating defects and Orkambi (a “dual” combination of Ivacaftor and Lumacaftor, a C1 corrector) for patients homozygous with the F508delCFTR mutation by Vertex Pharmaceuticals (Van Goor et al., 2011); (Orkambi). Recently another “dual” combination of Ivacaftor and Tezacaftor has been marketed by Vertex as Symdeko(Symdeko, 2018). Both of these “dual” combination therapies have shown only modest improvements of lung function (%FEV1) in patients homozygous for F508delCFTR mutations. In addition to being a cytochrome CYP3A4 inducer ((Report, 2015); (info, 2015)), Orkambi has been reported to demonstrate bronchoconstriction in some patients, most likely due to Lumacaftor’s off-target effects (Elborn et al., 2016; Hubert et al., 2017; Marigowda et al., 2017). These liabilities were not observed with Tezacaftor/Ivacaftor “dual” combination clinical studies, but still only modest lung function improvements (%FEV1) in addition to the reduced pulmonary exacerbations were observed for individuals that were homozygous with F508delCFTR or heterozygous with a second variant having residual function (Rowe et al., 2017; Taylor-Cousar et al., 2017). The C1 correctors target early folding of the mutant CFTR protein and could be combined with C2-type correctors with complementary mechanisms of action to deliver increased levels of F508del-CFTR protein to the plasma membrane. A triple combination of two correctors and a potentiator is expected to attain greater clinically benefits (Figure 1). Proof-of-concept for such triple combination small molecule therapy to yield an efficacious readout was recently demonstrated by Vertex Pharmaceuticals in their Phase 1 and 2 clinical trial results ((Taylor-Cousar, 2018); (Davies, 2018)); and subsequent results from their Phase 3 clinical trials with one triple combination with VX-659 (Davies et al., 2018) and the other with VX-445 (Vertex, 2019).

We would like to report the identification and characterization of ABBV-2222/GLPG2222 (herein referred as ABBV-2222; Figure 2), a C1 CFTR corrector, with significant improvements to potency and efficacy *in vitro* compared to currently available C1 correctors Lumacaftor and Tezacaftor. C1

Compounds like ABBV-2222, likely operate in a similar mechanism as described for Lumacaftor and possibly Tezacaftor and will be needed for novel triple combination therapies in addition to the ones coming on the basis of Tezacaftor. It possesses excellent potency and efficacy as compared to other C1 correctors in HBE TECC functional assay; however, there will still be need for efficacious triple combination CFTR modulators. Subsequent analysis of the drug properties of this molecule, like PK and other preclinical characterization showed it to be a suitable candidate for clinical development and hence advanced into clinical trials.

Materials and Methods

Cell Surface Expression-Horse Radish Peroxidase (CSE-HRP) Assay

Human lung derived epithelial cell line (CFBE41o-) (Ehrhardt et al., 2006) were used to develop a cellular assay for measuring the F508del CFTR cell surface expression after correction with test compounds. This was achieved by expressing the F508del CFTR mutation along with a horseradish peroxidase (HRP) in the fourth exofacial loop. The HRP activity was then measured using luminescence readout from these cells; CFBE41o-F508del CFTR-HRP, that were incubated overnight with the test corrector compounds (Veit et al., 2014). Briefly, for this primary assay, the CFBE41o-F508del CFTR-HRP cells were plated in 384-well plates (Greiner Bio-one; Cat 781080) at 4,000 cells/well along with 0.5 µg/mL doxycycline to induce the F508del CFTR-HRP expression and further incubated at 37 ^{o}C, 5% CO_2 for 72 hours. The test compounds were then added at the required concentrations and further incubated for 18-24 hours at 33 °C. The highest concentration tested was 20 µM with an 8-12 point concentration response curve using a 3-fold dilution. Three replicate plates were run to determine one EC_{50}. The EC_{50} values are represented as pEC_{50} values, defined as the negative log of EC_{50} values. All plates contained (dimethyl sulfoxide, DMSO) as negative controls and single concentration of (2-3 µM of 3-[(2R,4R)-4-([[1-(2,2-difluoro-1,3-benzodioxol-5-yl)cyclopropyl]carbonyl]amino)-7-methoxy-3,4-dihydro-2H-chromen-2-yl]benzoic acid) (Compound 15) (Wang et al., 2018) as positive controls; as well as on-plate concentration response of the positive control. The plates were washed 5X times with Dulbecco's phosphate buffered saline (DPBS) post incubation, followed by the addition of luminol (50 µL), as the HRP substrate. This was followed by measuring the HRP activity using luminescence readout on EnVision® Multilabel Plate Reader (Perkin Elmer; product number 2104-0010). The raw counts from the experiment are analyzed using Accelrys® Assay Explorer *v3.3*.

The % activity measured at each of the 8 test concentrations of the test compound was normalized to the on-plate positive control using the following formula:

% activity = [(test compound response – DMSO response) / (positive control response – DMSO response)]*100

The maximum % activity achieved for the test compound at any tested concentration is presented along with the pEC_{50} calculated using the general sigmoidal curve with variable Hill slope equation.

Western Blot Analysis of F508delCFTR Band C/B in CFBE Cells

CFBE cells stably expressing F508delCFTR (clone DG3) were obtained from the lab of Dr. Bob Bridges (Rosalind Franklin University, Medical School, USA) and were grown in MEM (Gibco), supplemented with 10% fetal bovine serum (Hyclone), 1% Pen/Strep and 500 µg/ml G418 (Gibco), at 37°C in a 5% CO_2 humidified incubator. CFBE cells were seeded at 10^6 cells per well onto 6-well dishes overnight and then treated with either DMSO or the indicated compound for 18-24 hours at 37°C in a 5% CO_2 humidified incubator. Prior to lysis, cell monolayers were rinsed twice with cold PBS to remove serum and medium. Cell plates were put on ice and lysed with the addition of RIPA buffer (Sigma) containing protease inhibitors (Roche). Lysates were centrifuged at 12,000 x g for 10 minutes at 4 ^oC to pellet insoluble material and total protein concentration was measured using the BCA protein assay (ThermoFisher). Equal amounts of protein were electrophoresed on NuPAGE® Novex 7% tris-acetate gels for 2 hours at 150V. Proteins were then transferred onto PVDF membranes at 20V for 1 hour using a Novex® Semi-Dry Blotter (Invitrogen). Membranes were incubated overnight at 4 ^oC with primary mouse monoclonal anti-CFTR 596 antibody (1:5000; University of North Carolina, Chapel Hill, NC; UNC596). Secondary antibody (IRDye 800CW goat anti-mouse IgG (1:15,000; LI-COR) was incubated for 1 hour at room temperature in the dark. Immunoblots were scanned on a LI-COR Odyssey infrared imaging

system and quantified using the system software. Mouse monoclonal α-Na/K ATPase antibody (1:5000; Abcam) was used to normalize sample loading.

Western Blot Analysis of F508delCFTR Band C/B in BHK Cells Expressing Suppressor Mutations

Suppressor mutations were used as a tool for a structural based corrector screen to identify correctors that either stabilizes the NBD1 and/or the NBD1-MSD2 interface of F508del CFTR protein based on the possible synergism. The two of the suppressor mutant variants used in these studies were R1S (G550E, R553Q, R555K and F494N mutations that stabilize the NBD1 domain) and R1070W (which stabilizes the NBD1-MSD2 interface).

Baby hamster kidney (BHK-21) cells, stably expressing the R1S or R1070W F508del CFTR with three tandem hemagglutinin-epitopes (3HA) in the fourth extracellular loop, were obtained from the lab of Dr. Gergely Lukacs (McGill University, Canada) and maintained as described previously (Sharma et al., 2004; Du et al., 2005). The HA tags preserved the functional and biochemical characteristics of CFTR (Haardt et al., 1999). BHK cells were grown in DMEM/F-12 (Gibco), supplemented with 5% fetal bovine serum (Hyclone) and 500 µM methotrexate (Hospira), at 37°C in a 5% CO_2 humidified incubator.

Briefly, the BHK suppressor mutant cells were seeded at $1e^6$ cells per well onto 6-well dishes overnight and then treated with either 0.2 % DMSO or 1 µM ABBV-2222 for 18-24 hours at 37°C in a 5% CO_2 humidified incubator. Just prior to lysis, cell monolayers were rinsed twice with cold PBS to remove serum and medium. Cell plates were put on ice and lysed with the addition of radio immunoprecipitation (RIPA) buffer containing protease inhibitors (Complete protease inhibitor cocktail, EDTA-free; Roche). Lysates were centrifuged at 14,000 x g for 15 minutes at 4 ^oC to pellet insoluble material and total protein concentration was measured using the BCA protein assay (Thermo). 50 µg of protein was electrophoresed on NuPAGE® Novex 3-8% tris-acetate

gels for 2 hours at 150V. Proteins were then transferred onto PVDF membranes at 20V for 1 hour using a Novex® Semi-Dry Blotter (Invitrogen). Membranes were incubated with primary antibody overnight at 4 ºC to detect the HA tagged-CFTR (mouse monoclonal anti-HA antibody (1:5000; Covance Innovative Antibodies). Secondary antibody (IRDye 800CW goat anti-mouse IgG (1:15,000; LI-COR) was incubated for 1 hour at room temperature in the dark. Immunoblots were scanned on a LI-COR Odyssey infrared imaging system and quantified using the system software. Mouse monoclonal anti-α-1 Na/K ATPase antibody (1:5000; Abcam; Cat ab7671) was used to normalize sample loading.

Correction of CFTR protein through action on membrane-spanning domain 1

HEK293 cells from the American Type Culture Collection (Manassas, VA) were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 1% fetal bovine serum (Thermoscientific, Waltham, MA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco) at 37°C in an atmosphere of 5% CO_2. Cell transfections were performed using Effectene reagent (Qiagen, Valencia, CA). The empty pcDNA3.1(+) vector was used to ensure equal micro-gram quantities of DNA were used in all transfection reactions.

Trans-epithelial Current Clamp (TECC) Assay using CFBE41o- (clone DG3) or primary Human Bronchial Epithelial Cells

A cell based assay was developed using the CFBE41o- (clone DG3) of the primary human bronchial epithelial cells (hBE) and used as a secondary assay to test novel F508del CFTR correctors for their activity on primary hBE cells with F508del/F508del CFTR mutation.

CFBE-DG3 Cells

CFBE41o- cells stably expressing F508del CFTR (clone DG3) were obtained from the lab of Dr. Bridges, Rosalind Franklin University and cultured in MEM (Gibco) supplemented with 10% fetal

bovine serum, 1% penicillin/streptomycin and G418 selection (0.5 mg/ml) in a humidified 5% CO2, 95% O2 incubator at 37 °C. For propagation, the CFBE41o- cells were cultured in plastic flasks coated with an extracellular matrix consisting of 10 µg/ml human fibronectin (EMD), 30 µg/ml PureCol collagen (Advanced Biomatrix) and 100 µg/ml BSA (Sigma-Aldrich) diluted in MEM basal medium. For TECC measurements, CFBE41o- cells were seeded onto collagen coated 24 well filters at a density of 1.5 x 10^5 cells/filter and cultured for 5 -7 days in the above growing media to form a tight monolayer.

Human Bronchial Epithelial Cells

Primary human bronchial epithelial (hBE) cells from CFTR patients with homozygous F508del/F508del mutation were expanded from 1×10^6 to 250×10^6 cells (Neuberger et al., 2011). For this purpose, cells isolated from CF patients with the homozygous mutation, procured from the CF center tissue procurement and cell culture core at the Marsico Lung Institute at UNC (Randell) as well as the Cystic Fibrosis translational research center at McGill University (University) were seeded onto 24 well Corning (Cat # 3378) filter plates that were coated with 3T3 conditioned media and grown at an air-liquid interface for 35 days using an Ultroser® G supplemented differentiation media. All the primary human bronchial epithelial cells were collected in accordance with IRB approval protocols. Apical surface mucus was removed 72 hours before the experiment by incubating the apical surface of the cells for 30 minutes with 3 mM dithiothreitol (DTT) prepared in DPBS with Ca^{2+} and Mg^{2+}.This was followed with aspiration of the mucus from the apical surface along with DPBS. The apical surface is re-washed with phosphate buffered saline (PBS) incubated for 30 minutes followed with aspiration. These HBE cells were then incubated with the desired concentrations of the corrector compounds 18-24 hours at 37 ºC, 5% CO_2. The desired concentrations of the corrector compounds were prepared from the 10 mM stocks in differentiation media and were always applied on the basolateral side of the epithelial cells. We also used an assay format where a fixed concentration of potentiator was added

chronically along with the corrector compounds. This chronic treatment with the potentiator helps eliminate any interaction that might happen with CFTR modulators (Cholon et al., 2014; Veit et al., 2014) (correctors and potentiators) and thereby determine the true efficacy of the modulator combinations reflective of clinical relevance.

TECC (Transepithelial Current Clamp) Assay

The assay uses a Transepithelial Current Clamp (TECC) (Vu et al., 2017) instrument that can measure the functionality of the mutated channel by measuring the equivalent CFTR current (I_{EQ}) generated by the polarized primary epithelial cells. We used a TECC-24 with a 24 channel electrode manifold allowing for the simultaneous measurement of the transepithelial voltage, V_T, and transepithelial resistance, R_T, under current clamp conditions, from 24 filters using a 24 well Costar filter plate. The design of the filters in the 24 well filter plates is exactly the same as the design of an individual Transwell filter used in the classical Ussing Chamber with a surface area of 0.33 cm^2. Each measured V_T values are corrected for the electrode offset potential measured using buffer alone in a separate plate, and each measured R_T values are then corrected for the combined solution series and empty filter resistances. The corrected V_T and R_T values were then used to calculate the equivalent current, I_{EQ} using Ohm's law ($I_{EQ} = V_T/R_T$). The area under the curve (AUC) for the time period between the forskolin peak I_{EQ} response and at the time of bumetanide addition was also calculated using a one-third trapezoid method, in addition to calculating the I_{EQ}. Unlike the FRT cells where the expression of CFTR is not polarized, expression of CFTR in the CFBE cells is restricted to the apical membrane, and in cells over expressing CFTR, the Gt (the transepithelial conductance) response largely reflects apical membrane conductance changes. When using CFBE cells in the TECC, Gt was measured using a custom micro-controlled transepithelial current clamp (TECC). Cells were simulated with a 30 mV sine wave at a frequency of 1 Hz and the current response sampled at 8 kHz. Gt was calculated using Ohm's law where $Gt = \Delta I/\Delta Vt$ (ΔI is the delta current response to the 30 mV

voltage sine wave, ΔVt). The assay was run in a 24-well format and all 24-wells were measured at the same time point giving a higher throughput for this assay. On the day of measuring the corrector activity on the TECC, the cells were switched into a bicarbonate and serum free F-12 Coon's medium and allowed to equilibrate for 180 minutes for the CFBE-DG3 cells and 30 minutes for HBE cells in a CO_2 free incubator. At the time of measurement, the apical and basolateral sides of the filter were bathed with the F-12 Coon's modification media (with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 (using 1 M tris(hydroxymethyl)aminomethane (Tris)), and the measurements were made at 36.5 ^{o}C. Current responses before and after the sequential addition of benzamil (apical 6 µM addition; for inhibiting epithelial ENaC channel), forskolin (apical and basolateral 10 µM addition; for activating the CFTR channel), control potentiator, (N-(3-carbamoyl-5,5,7,7-tetramethyl-4,7-dihydro-5H-thieno[2,3-c]pyran-2-yl)-1H-pyrazole-5-carboxamide (GLPG1837) (Van der Plas et al., 2018); apical and basolateral 1 µM addition; for potentiating the CFTR channel) and bumetanide (basolateral 20 µM addition; for inhibiting the Na:2Cl:K co-transporter, an indirect measure of inhibiting the Cl-secretion driven by CFTR channel) were measured.

All plates contained negative controls (dimethyl sulfoxide, DMSO) which coupled with the control potentiator (N-(3-carbamoyl-5,5,7,7-tetramethyl-4,7-dihydro-5H-thieno[2,3-c]pyran-2-yl)-1H-pyrazole-5-carboxamide) sets the null response and positive controls (3 µM of 3-[(2R,4R)-4-([[1-(2,2-difluoro-1,3-benzodioxol-5-yl)cyclopropyl]carbonyl]amino)-7-methoxy-3,4-dihydro-2H-chromen-2-yl]benzoic acid) (Compound 15) (Wang et al., 2018) coupled with the control potentiator sets the 100% response to measure the correction of the mutated CFTR channel. The maximum percent activity (Emax) is reported relative to the positive control value.

The % activity measured at each of the 6 test concentrations of the test compound was normalized to the on-plate positive control using the following formula:

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% activity = [(test compound response – DMSO response) / (positive control response – DMSO response)]*100

The I_{EQ} and AUC at different test concentrations were fit and an EC_{50} was calculated using the general sigmoidal curve with variable Hill slope equation included in the Prism *v5* software.

Patch-clamp Electrophysiological Assay

To examine the open probability (P_o) of F508delCFTR after pretreatment with ABBV-2222, CHO cells transiently transfected with CFTR-cDNA (pcDNA 3.1 Zeo (+) vector; Invitrogen, Carlsbad, CA, USA) and green fluorescent protein encoding pEGFP-C3 (Takara Bio Inc., Shiga, Japan) were incubated with ABBV-2222 overnight before patch-clamp experiments. To avoid repetition, detailed experimental methods, materials and data analysis can be found in our latest publication (Yeh et al., J. Physiol. 2019).

Chemicals and Reagents

Benzamil and Forskolin were purchased from Sigma-Aldrich. The selective CFTR inhibitor, $CFTR_{Inhib}$-172, was purchased from Tocris Biosciences. The Na-K-2Cl cotransporter inhibitor, bumetanide, was purchased from Sigma-Aldrich. Vertex CFTR modulators, Lumacaftor, Tezacaftor and Ivacaftor were purchased from SellekChem. All Abbvie/Galapagos CFTR modulators were synthesized at Abbvie or Galapagos and these test compounds were tested from a 10 mM stock solution made with DMSO and stored at room temperature.

CFTR expression plasmids pcDNA3.1(+)-CFTR and pcDNA3.1(+)ΔF508-CFTR have been described elsewhere (Meacham et al., 2001; Younger et al., 2006). CFTR constructs representing CF disease–causing point mutants or truncated biogenic intermediates were made using the QuikChange protocol (Stratagene, Santa Clara, CA). The CFTR antibody used in this study was MM13-4 (N-terminal tail epitope) from Millipore, Darmstadt, Germany.

Statistical Analysis

Results are expressed as means ± SD or SEM. of n observations. Statistical analysis was carried out using GraphPad Prism (GraphPad Software, La Jolla, CA) *v* 5.0 for Windows.

Results

Identification of an active chemotype and ABBV-2222 using a cell surface expression assay

The first-in-class first generation of C1 correctors, Lumacaftor and Tezacaftor were developed and marketed by Vertex Pharmaceuticals. We have identified and developed a novel C1 corrector, ABBV-2222, that has a differentiated structure with improved potency and enhanced clinical characteristics (Wang et al., 2018). Figure 2 shows the structure of these correctors and corrector Compound 15, a precursor of ABBV-2222 used as the control in our biological assays, as well as A-9433 which is the inactive enantiomer of ABBV-2222; and the structure of Vertex marketed CFTR potentiator, Kalydeco (VX-770 or herein referred as Ivacaftor) along with GLPG1837, the potentiator used in our biological assays described in this manuscript. Figure 3a shows the medicinal chemistry process that started from the hit identified from a high-throughput primary screen. The virtual libraries were enumerated from selected proprietary acid and amine monomers present in Abbvie's collection to assemble novel lead compounds through a final amide bond coupling reaction. The final products were then filtered based upon optimized chemical and physiochemical properties and actual compounds synthesized for their structure-activity-relationship (SAR). After initial SAR to identify sets of active compounds, an empirical based SAR effort was utilized for optimizing compound potency, its clearance and other drug-like properties. The high throughput Cell Surface Expression-Horse Radish Peroxidase (CSE-HRP) assay for measuring the F508del CFTR cell surface expression after correction with test compounds was utilized to identify the chemotype and all the further primary SAR studies. The medicinal chemistry cycle of compound synthesis and SAR analysis was repeated to refine and finally generate potent lead compounds with good drug-like properties. ABBV-2222 was identified from these set of lead molecules. A plot representing the potency and efficacy improvement progression through understanding of SAR of the compounds is also shown in

Figure 3a. Concentration response for membrane expression efficacy of ABBV-2222 relative to Lumacaftor and Tezacaftor as measured by the CSE-HRP assay is shown in Figure 3b. ABBV-2222 was approximately 10- and 20-fold more potent than Lumacaftor and Tezacaftor respectively. It had an EC_{50} of 27 nM; pEC_{50} 7.56 ± 0.16 (n=19), compared to 251 nM; pEC_{50} 6.6 ± 0.08 (n=3) and 586 nM; pEC_{50} 6.23 ± 0.05 (n=6) for Lumacaftor and Tezacaftor respectively. The relative efficacies from these experiments were 141 ± 15 (n=19), 136 ± 5 (n=3) and 105 ± 14 (n= 6) for ABBV-2222, Lumacaftor and Tezacaftor respectively. Efficacy drop-off was observed for ABBV-2222 above 2.22 µM and Lumacaftor above 6.67 µM (data not shown in the plot and not used for EC_{50} calculations). Compound 15 showed an EC_{50} of 230 nM with a relative efficacy of 101% in this assay and was subsequently used as an on-plates control for our internal biological assays.

Biochemical and Functional characterization of ABBV-2222

ABBV-2222 promotes maturation of F508delCFTR as assessed by Western Blot

Biochemical characterization to assess the effects of ABBV-2222 on the maturation of F508delCFTR mutated protein was performed in the CFBE41o- cells stably expressing F508del CFTR (CFBE clone DG3) that were obtained from the lab of Dr. Robert Bridges, Rosalind Franklin University. Western Immunoblots are presented in Figure 4a-b and α1 Na^+/K^+ ATPase was used as a loading control. Consistent with the observations in the CSE-HRP assay, ABBV-2222 promoted maturation of F508delCFTR as measured by the accumulation of a higher molecular weight glycosylated band (Band C) by Western blot analysis (Figure 4a). A concentration-dependent increase of the mature Band C was observed, similar to the one observed with Tezacaftor albeit at lower concentrations (Figure 4b). Bar graph showing mean with SD obtained from three independent experiments (n=3) of the Band C/B ratio for the representative Western blot experiment shown in Fig. 4a.

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Functional I_{EQ} Measurement Using TECC-24 in CFBE and Primary hBE Cells and Po measurement Using Single channel excised Patch clamp

To determine if restoration of surface expression is translated to a functional response, we measured the forskolin-activated Cl^- conductance in these CFBE clone DG3 cells using the semi-automated TECC-24 instrument. As shown in Figure 4c, the increase of F508del-CFTR rescue by ABBV-2222 as monitored by the function, measured as an increase in conductance (Gt) due to CFTR channel opening on the membrane facilitated by a potentiator, was concentration-dependent, with an EC_{50} of 20.8 ± 2.7 nM (SEM; n=6 at each concentration). Analogous to the efficacy difference observed in the Western blot results, Tezacaftor was ~14-fold less potent than ABBV-2222, with an EC_{50} of 266.9 ± 38.6 nM (SEM; n=6 at each concentration).

Further functional characterization of ABBV-2222 was extended to more biologically and clinically relevant primary human bronchial cells derived from the CF patients homozygous for F508del variant. The cultured F508del-HBE cells were incubated with ABBV-2222 for 24 hours prior to the electrophysiological readout and referred to as a “chronic treatment” with the compounds at the indicated test concentrations and treatment duration. CFTR-mediated equivalent current (I_{EQ}) from hBE cells was measured using TECC-24 in the continuous presence of corrector or potentiator compounds. Figure 5a shows representative concentration-response curves for ABBV-2222 in comparison with Tezacaftor and Lumacaftor, with an EC_{50} (in nM) of 2.85 ± 0.4 (SEM; n=5 different donors), 148.2 ± 25.7 (SEM; n=4 different donors) and 122.8 ± 13.8 (SEM; n=1 donor) respectively. GLPG1837 was used as the potentiator in these experiments and was added acutely in combination with Forskolin (10 µM). ABBV-2222 also exhibited equivalent efficacy to Lumacaftor, and ~20% improvement in efficacy compared to Tezacaftor as measured with this functional assay in a dual format. Taken together, ABBV-2222 differentiates itself at the level of *in vitro* potency (from both Lumacaftor and Tezacaftor) and

efficacy (from Tezacaftor). Potency ranges of ABBV-2222 were determined in HBE TECC assay from 16 different CF patient derived donor cells that were homozygous for the F508delCFTR variant. As shown in Figure 5b, the TECC functional potencies ranged from 1.6 nM to 13.1 nM across these homozygous 16 F508delCFTR variant donors' cells with the median potency around 6 nM. Enantiomeric selectivity was demonstrated by A-9433, an enantiomer of ABBV-2222, which was ~160-fold less potent and ~70% less efficacious than ABBV-2222 (data not shown). ABBV-2222 does not show additivity with Lumacaftor or Tezacaftor in the hBE TECC functional assay and likely works via a similar mechanism of action. Representative example for lack of functional additivity for Lumacaftor and ABBV-2222 is shown in Figure 5c. Emax concentrations for both Lumacaftor and ABBV-2222 were chronically incubated along with GLPG1837 (0.1 µM) as a potentiator for this experiment (n=8 replicates for each condition). As shown in Figure 5d, there was no difference in potency of ABBV-2222 in both acute and chronic mode of addition of the potentiator (EC_{50} 7.9 nM *vs.* 7.3 nM respectively). The potencies were determined using one primary HBE donor with the F508delCFTR variant, with n=3 replicate values at each tested concentration. Potentiator GLPG1837 does not have any non-productive interactions with the correctors for their relative efficacy in the Western blot protein measurement assay or the TECC functional assay. And, to have a more clinically relevant format, all the compounds were added chronically and are added back so they are also present during all the TECC experimental runs. There is no change in potency of ABBV-2222 in the absence or presence of GLPG1837 potentiator (0.1 µM) (EC_{50} 6.4 nM *vs.* 5.4 nM respectively) as shown in the concentration-response curves in Figure 5e. The potencies were determined using one primary HBE donor with the F508delCFTR mutation, with n=2 replicate values at each tested concentration. We also determined the onset of F508delCFTR activity in the functional HBE TECC assay. Similar to previous report (Lukacs et al., 1993), the functional response showed rapid onset with maximal effect achieved within 12 hrs. of treatment, as well as sustainability up to 96 hours in the TECC system (Figure 5f).

To assess the channel function of F508delCFTR pretreated with ABBV-2222, patch-clamp experiments in the inside-out mode were performed in the CHO cells expressing F508delCFTR. Figure 6 shows a real-time current recording of F508delCFTR which has been activated by protein kinase A and ATP (upper trace). There are only 5 distinguishable openings in > 1 minute of recording in the presence of ABBV-2222, suggesting a persistent gating defect not corrected by ABBV-2222. Similar recording was obtained in the absence of ABBV-2222 (data not shown). However, in the same patch, the addition of the CFTR potentiator GLPG1837 (Yeh et al., 2017), (Yeh et al., 2019) dramatically increased the activity to reveal the presence of at least 8 F508delCFTR channels in the patch (lower trace in Figure 6). Single-channel kinetic analysis of data like this revealed an apparent P_o of 0.57 in in the presence of GLPG1837, whereas the P_o of ABBV-2222-treated F508delCFTR was 0.04, which was similar to the vehicle treated control reported previously (Kopeikin et al., 2014).

ABBV-2222 Stabilizes Nucleotide Binding Domain 1 - Membrane Spanning Domain interface

Several investigators have successfully demonstrated the Lumacaftor target F508del-CFTR during endoplasmic reticulum biogenesis and engage in undefined interactions to facilitate protein maturation. As well documented in literature, and shown in Figure 7a, F508del mutation in CFTR causes severe dysregulations of NBD1 energetics as well as domain-domain interactions, mainly the NBD1-MSD1/2 interface. It has been previously shown that stabilization of NBD1 or NBD1-MSD2 interface by second site suppressor mutations such as R1S or R1070W only modestly improves CFTR folding (< 20%). However, simultaneous stabilization of these primary structural defects can lead to robust rescue of CFTR folding (~80%) (Okiyoneda et al., 2013). CFTR-F508del integrated with the R1070W suppressor mutation has been shown to stabilize the NBD1-MSD2 interface and hence serves as a reference of how Type C1 correctors function. Type C2 correctors promote the stabilization of the NBD2 interactions, and

Type C3 correctors stabilize NBD1 directly (Okiyoneda et al., 2013). CFTR-F508del integrated with the R1S suppressor mutations have been shown to stabilize NBD1 directly and replicates the actions of a Type 3 corrector. Okiyoneda *et al* have demonstrated that compounds like Lumacaftor preferentially target the NBD1-MSD1/2 interface, and hence classified them as Type C1 corrector.

Suppressor mutants were used in our study as a tool for a structural based characterization of our C1 corrector, ABBV-2222 in order to determine if it stabilizes the NBD1 domain and/ or the NBD1-MSD2 interface, based on the possible synergism with R1S or R1070W. As shown in our WB data, ABBV-2222 substantially rescued the folding of F508del-CFTR-R1S and it had a lesser impact on F508del-CFTR-R1070W, respectively. This suggests that ABBV-2222 preferentially impacts the NBD1/MSD2 interface restoration over the NBD1 structural defects, and hence can be classified as a Type C1 corrector that functions by promoting stabilization of the interface between Nucleotide Binding Domain 1 (NBD1) and the Membrane Spanning Domain 2 (MSD2). ABBV-2222 exhibits more efficiency in restoring Band C formation with BHK cells expressing the R1S suppressor mutation compared to those expressing the R1070W suppressor mutation. The pattern of Band C/B ratio is similar to that reported for Lumacaftor, supporting the idea that ABBV-2222 stabilizes the NBD1-MSD2 interface as a Type C1 corrector.

In analogy with this suppressor mutant model, corrector pairs acting on different primary structural defects are needed to restore F508del-CFTR folding synergistically and those will be the ones that will yield better efficacy. As both primary structural defects need to be corrected in order to notice a substantial rescue of F508del-CFTR folding, a small molecule acting on the same pre-stabilized intramolecular interdomain interaction (by R1S suppressor mutation) is unlikely to produce the level of correction observed in our WB data for R1S.

ABBV-2222 was selective for correction of F508delCFTR processing and was unable to improve the processing of other misfolded mutant proteins like P-glycoprotein (G268V-PgP) and hERG K+ channel (G601S-hERG) (data not shown).

ABBV-2222 Corrects F508delCFTR through action on Membrane Spanning Domain 1

Another set of experiments were conducted to further explore whether ABBV-2222 behaved in a similar fashion as Lumacaftor and Tezacaftor which corrects the folding defects in CFTR protein via a direct action on a specific region of CFTR, *i.e.*, on the Membrane Spanning Domain 1 (MSD1), and this correction does not require the presence of NBD1. Biochemical assays monitoring the effects of CFTR modulators on the steady-state accumulation and half-life durations of different lengths of CFTR fragments have been used to study CFTR biogenesis. Ren *et al* (Ren et al., 2013) have demonstrated that the shortest-length CFTR requirement for Lumacaftor action is on the N-terminal CFTR fragment that only contained MSD1 (Figure 8a). We saw similar protein fragment correction pattern with ABBV-2222, so the shortest-length of CFTR fragment affected by ABBV-2222 was CFTR 375, which contains only MSD1 (Figure 8b). Like Lumacaftor, ABBV-2222 did not increase the stability of CFTR 837-1480 (data not shown). As shown in Figure 8b, ABBV-2222, Lumacaftor and Tezacaftor were all unable to increase the accumulation of CFTR 370, suggesting that they do not act as a proteasome inhibitor, whereas, Bortezomib was able to increase the stability of this CFTR fragment (Ren et al., 2013). Cycloheximide-chase experiments were carried out to determine the half-life of CFTR 380 fragment in the presence of protein synthesis inhibitor cycloheximide (10 μg/ml) and changes in CFTR 380 signals over the course of 2-hour chase reaction were normalized to the CFTR 380 signal at T=0), as shown in Figure 8c. The half-life of CFTR 380 was increased in the presence of ABBV-2222, Lumacaftor or Tezacaftor, as compared with untreated control, indicating that the increase in the steady-state accumulation caused by these compounds is associated with an

increase in the stability of the CFTR 380 fragment. In summary, ABBV-2222 acts similar to Lumacaftor and Tezacaftor, with improved stabilization of CFTR 375.

Discussion

The molecular defect caused by different CFTR variants, especially F508delCFTR, has been a focus of studies that have shown that small molecules are able to enhance the amount and function of CFTR at the cell surface in different cell systems, including the primary hBE, hNE and intestinal organoids (Awatade et al., 2018). The past two decades have seen an exponential growth in the discovery and development of these small molecule CFTR modulators, namely the CFTR potentiators and CFTR correctors. Indeed one of the biggest breakthroughs in the field of CF this decade has been the development and marketing of CFTR potentiators and CFTR correctors that rectify the underlying defects in the CFTR protein (Hongyu et al., 2017; Gentzsch and Mall, 2018). Referred as the first generation CFTR C1 correctors, both Lumacaftor and Tezacaftor restored F508delCFTR folding and increased the CFTR function in preclinical *in vitro* testing in combination with a CFTR potentiator, Ivacaftor to ~25% normal CFTR activity (Van Goor et al., 2011). Monotherapy in CF carrying homozygous F508delCFTR variant with both of these C1 correctors did not show marked improvement in lung functions as measured by FEV_1 (Clancy et al., 2012). Even the dual therapy of either of these C1 correctors in combination with a potentiator, Ivacaftor, only resulted in a significant but marginal improvement in lung function, in the range of ~3-4% FEV_1 (Wainwright et al., 2015); (Ratjen et al., 2017). Based upon their objective of meeting the primary endpoint of statistically significant FEV_1 improvement, both Orkambi (Lumacaftor plus Ivacaftor) and Symdeko (Tezacaftor plus Ivacaftor) have been approved by the FDA for the treatment of CF patients with homozygous F508delCFTR variant. However, adverse respiratory events resulting in a discontinuation rate of 25-30% in the first three months that is largely attributed to Lumacaftor have been reported for the patients on Orkambi(Hubert et al., 2017); (Jennings et al., 2017); (Labaste et al., 2017). In addition, a noteworthy drawback of Lumacaftor is its ability to cause strong cytochrome P450-3A induction that limits its use in patients with Ivacaftor-responsive variants, even F508delCFTR (info, 2015).

Tezacaftor was developed closely behind Lumacaftor to address the liabilities described above (Van Goor, 2016). ABBV-2222 represents a novel C1 CFTR corrector discovered by AbbVie in collaboration with Galápagos, distinguished from other C1 correctors on the market, with significant improvements in potency and drug-drug-interaction (DDI) (Wang et al., 2018), Ref 60) profile compared to Lumacaftor and improvements in potency and efficacy compared to Tezacaftor. Based upon these characteristics, ABBV-2222 has been advanced into clinical trials.

Data presented here indicates that ABBV-2222 acts via a similar mechanism of action as Lumacaftor and Tezacaftor in promoting proper folding of the F508delCFTR variant in the ER to facilitate trafficking to the cell surface. Though the structure of ABBV-2222 resembles Lumacaftor and Tezacaftor, the difluoromethoxy chromane with the phenyl group is a unique chemical entity that endows a high potency not achieved by the other reported C1 correctors (Altenbach et al., 2016). Several lines of investigation were used to demonstrate the effectiveness of ABBV-2222 to correct the misfolded F508delCFTR protein for producing a functional protein on the membrane. The assays utilized with the background of the CFBE41o- epithelial cells were correlative of the corrector activities observed as measured with the different readouts, *e.g.* the potencies in the cell surface expression (CSE) cellular assay was correlative with the subsequent Western blot Band C and the TECC experiments of ABBV-2222 (as shown in Figure 4). Western blot experiments demonstrated that ABBV-2222 promoted maturation of F508delCFTR as measured by the accumulation of a higher molecular weight glycosylated band (Band C) in the CFBE cells, and this maturation was not affected by chronic co-incubation with the clinically relevant concentrations of the potentiator (data not shown). Similar observations were made with the functional readouts in the TECC assay, whereby the corrected F508delCFTR in the primary HBE cells homozygous for the same variant showed no difference in the potency of ABBV-2222 with either acute or chronic presence of clinically relevant

potentiator concentrations. This characteristic of ABBV-2222 uniquely separates it from Lumacaftor, for which several *in vitro* studies have shown a non-productive interaction in the presence of potentiator, Ivacaftor (Cholon et al., 2014; Veit et al., 2014). However, the high non-physiologically relevant concentrations used in these studies do question the validity or significance for such interactions in a clinical setting ((Matthes et al., 2016)). We did not observe any such interactions for ABBV-2222 with any of the potentiators at their clinically relevant concentrations. ABBV-2222 appears to work purely as a corrector/trafficking enhancer. ABBV-2222 promotes an increase in the amount of F508delCFTR protein at the plasma membrane as measured by Western Blot, and this corrected protein was functional, *albeit* minor, in the TECC assay in the absence of a potentiator. However, there was no observed increase in the open probability (P_o) of corrected CFTR as measured with the single channel excised patch experiments after the overnight treatment (as shown in Figure 6), suggesting that observed increase of activity in functional HBE TECC measurements is due to the presence of increased amounts of F508delCFTR at the plasma membrane and not due its improved channel activity. Subsequent acute addition of a potentiator in these experiments resulted in a P_o similar to that a *wt* CFTR protein (Lin et al., 2016), suggesting ABBV-2222 promotes a properly folded channel gating activity for F508del-CFTR in the presence of a potentiator. Therefore, it appears that the fraction of F508delCFTR protein that ABBV-2222 is able to export out of the ER attains a more stable protein conformation and suggests it is not recognized as improperly folded by the membrane peripheral quality control pathways (Okiyoneda et al., 2010). This is further supported by the normal functional onset of the corrected F508delCFTR in the primary HBE TECC assay (as shown in Figure 5f). The relative functional efficacy of our dual combination (ABBV-2222 plus potentiator GLPG1837) is equivalent to that of Lumacaftor plus Kalydeco, and approximately 30% better than that of Tezacaftor plus Kalydeco combination, in side-by-side comparison in the HBE TECC assay using homozygous F508delCFTR HBE cells (as shown in Figure 5a).

Using the targeted biosensor technology of Sharpedge Labs (Gentzsch and Mall, 2018) to study the cellular trafficking of misfolded proteins, we were able to demonstrate the selectivity of ABBV-2222 for the processing of F508delCFTR. As compared to the efficient folding detected for F508delCFTR, ABBV-2222 was unable to process mutant hERG (G601S) and mutant PgP (G268V). VRT-325, a positive control in both of these assays was not selective and it increased the processing of all the proteins tested (Van Goor et al., 2011).

Several investigators have successfully shown the discovery and development of novel CFTR modulators (Balut et al., 2017; Grootenhuis et al., 2017; Hongyu et al., 2017; Gentzsch and Mall, 2018) that are biochemically and functionally additive to the C1 corrector class of F508delCFTR modulators, *e.g.*, Lumacaftor, Tezacaftor and ABBV-2222. Development of ABBV-2222 represents a novel C1 corrector as the first component of the triple combination therapy being developed by Abbvie. It remains a challenge to attain a clear understanding of the causal relationship that exists between the three components of this triple combination therapy. Ongoing efforts (Hongyu et al., 2017) are focused on understanding the mechanism of action (MoA) and drug-drug interactions of these three components to produce a best-in-class, clinically safe and efficacious triple combination therapy for the CF patients.

Using C1 correctors as the foundation of triple agent combination therapies, coupled with the recent proof-of-concept studies demonstrated by Vertex (Davies, 2018); (Taylor-Cousar, 2018), there is the potential to treat all patients who harbor at least one copy of the F508del mutation (~ 90% of patients). However, there are still patients with known heterozygous missense variants that are not responsive to this triple combination therapy. This includes CF patients with nonsense mutations, leading to premature termination as well as the less characterized RNA splicing mutations. These variants have been the focus of a major discovery effort led by the North American CF Foundation at several labs (Clancy et al., 2018) and also by other individuals.

In sum, there has been tremendous progress in the field that continues in the pursuit of providing disease-changing therapies for all CF patients, regardless of the different variants they carry. Data presented in the current work lead us to propose that ABBV-2222 has distinct advantages over existing C1 correctors and could serve as a foundation for another potential triple combination therapy option for the CF patients.

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2. Footnotes

AKS, YF, CB, SA, AMM, AMS, YJ, TAV, BL, XBS, XW, WG, PK, and CT are employees of AbbVie. The design, study conduct, and financial support for the research conducted by AbbVie were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication. The design, study conduct, and financial support for this research were provided by AbbVie and Galapagos.

KC is an employee of Galapagos and may own stock from the company.

Figures Legends

Figure 1. CFTR Modulators. Total of 336 disease-related variants/mutations have been listed for the CFTR protein at https://www.cftr2.org/mutations_history. A complementary "triple combination" therapy consisting of two corrector molecules, type 1 (C1) and type 2 (C2) with additive mechanisms along with a potentiator (P) are being developed by several investigators for maximum restoration of mutated CFTR function.

Figure 2. Chemical structures of CFTR modulators discussed in the manuscript. Lumacaftor and Tezacaftor are the C1 class correctors, and Ivacaftor is the CFTR potentiator that are drugs marketed by Vertex Pharmaceuticals for treating Cystic Fibrosis. ABBV-2222 is a C1 class corrector that is currently in Phase II clinical trials; compound 15 is a precursor of ABBV-2222 that is used as on-plate control in the biological assays described in the manuscript and A-9433 is the inactive enantiomer of ABBV-2222. GLPG1837 is the potentiator of CFTR used in this manuscript.

Figure 3.

a. Medicinal chemistry process of Lead generation followed by the identification of ABBV-2222. Detailed structure-activity-relationship (SAR) data for the chemical series using the CSE-HRP assay showing a distribution of the potency and efficacy for ABBV-2222 relative to other derivatives as well as Lumacaftor and Tezacaftor.

b. Representative concentration-response of ABBV-2222 in the cell surface expression assay in comparison with Lumacaftor and Tezacaftor. Data shown as mean ± SD of 3 replicates from a representative experiment, however, several replicates were run as described below. ABBV-2222 was approximately 10- and 20-fold more potent than Lumacaftor and Tezacaftor respectively. It had an EC_{50} of 27 nM; pEC_{50} 7.56 ± 0.16 (n=19), compared to 251 nM; pEC_{50} 6.6 ± 0.08 (n=3) and 586 nM; pEC_{50} 6.23 ± 0.05

(n=6) for Lumacaftor and Tezacaftor respectively. The relative efficacies from these experiments were 141, 136 and 105 percent normalized to the on-plate compound 15 for ABBV-2222, Lumacaftor and Tezacaftor respectively. Efficacy drop-off was observed for ABBV-2222 above 2.22 µM and Lumacaftor above 6.67 µM (data not shown in the plot and not used for EC_{50} calculations).

Figure 4.

a. Three-point concentration response of ABBV-2222 and Tezacaftor on F508delCFTR maturation in the CFBE cells as reflected by band C. Compounds were incubated for 24 hrs. with the cells prior to harvesting and lysis.

b. Bar graph showing mean with SD from n=3 replicates of the Band C/B ratio including the representative Western blot experiment shown in Fig. 4a.

c. Concentration response of ABBV-2222 and Tezacaftor on F508delCFTR function in the CFBE-DG3 cells measured in the TECC assay in the presence of the potentiator GLPG1837. The EC_{50} for ABBV-2222 was 20.8 ± 2.7 nM as compared to 266.9 ± 38.6 nM for Tezacaftor (n= 6 replicates at each concentration tested).

Figure 5. Characterization of functional F508delCFTR correction by ABBV-2222 in HBE TECC assay

a. Concentration-response curve of ABBV-2222, Lumacaftor and Tezacaftor in TECC assay using primary HBE cells with homozygous for F508delCFTR and using potentiator GLPG1837. Compounds were added to the cells for 24 hrs. prior to the electrophysiological readout using TECC. The percent activity was compared to the activity of the control compound 15 in the presence of potentiator GLPG1837.

b. Scatter plot of HBE TECC potency of ABBV-2222 across 16 different F508del/F508delCFTR homozygous donors. The potencies ranged from 1.6 nM to 13.1 nM across 16 different primary HBE donor cells with the median potency around 6 nM.
c. Bar graph showing lack of functional additivity for Lumacaftor and ABBV-2222 in the TECC functional assay F508del/F508delCFTR homozygous donor (mean with SEM from n=8 replicates).

d. Concentration-response curve of ABBV-2222 to compare the potency of ABBV-2222 with the acute addition of GLPG1837 potentiator (the potentiator was added together with forskolin at time of TECC assay readout) or added chronically (co-incubation of GLPG1837 with ABBV-2222 during 24 hours prior to addition of forskolin for channel activation). The EC_{50} determined in the two conditions were similar, 7.9 nM (acute incubation) compared to 7.3 nM (chronic incubation)
e. HBE TECC potency of ABBV-2222 was determined with or without chronic addition of the potentiator GLPG1837
f. Onset of rescued F508delCFTR protein function was determined using the TECC assay after correction with ABBV-2222 in the presence of GLPG1837 at the indicated times (n=4; representative data from a single donor that was homozygous with F508delCFTR mutation). DMSO condition represents cells incubated with the potentiator GLPG1837 but without any corrector ABBV-2222.

Figure 6. Single channel open probability (Po) of F508delCFTR protein corrected with ABBV-2222 in the absence and presence of potentiator GLPG1837.

a. Patch clamp experiments were performed in CHO cells expressing F508delCFTR ((Lin et al., 2016)) to determine the open probability (Po) of the F508delCFTR protein in the absence (0.04) and presence (0.57) of potentiator GLPG1837.

b. Bar graph representation of the data shown in a (n=4 replicates). ABBV-2222 does not have direct affect the Po of the corrected F508delCFTR channel until activated by GLPG1837 potentiator.

Figure 7. ABBV-2222 functions as a Type C1 corrector

a. Proposed model categorizing different types of CFTR correctors.

b. Western blot analysis of CFTR ± 1 μM ABBV-2222 in BHK cells expressing either R1S-CFTR-F508del (R1S) or R1070W-CFTR-F508del (R1070W) suppressor mutations. BHK cells overexpressing either R1S or R1070 were incubated with ABBV-2222 for 24 hrs. Cells were harvested, lysed and loaded onto a 3-8% tris-acetate gel. CFTR was detected using CFTR antibody 596.

Figure 8. The impact of CFTR modulators on the accumulation of short CFTR fragments that contain MSD1 and different length C-terminal extensions.

a. Schematic diagram of CFTR fragments whose accumulation becomes sensitive to modulators upon inclusion of residues that are located between amino acid 375 and 380.

b. Comparison of ABBV-2222, Lumacaftor, and Tezacaftor on the steady-state accumulation of the indicated CFTR fragment. Indicated modulators were added at 5 μM to cultures of HEK293 cells 14 hours prior to harvest and detection of the fragment.

c. Modulators were added to cells that expressed 380X-CFTR at T=0 of the chase reaction in the same media with the protein synthesis inhibitor cycloheximide (10 μg/ml). The

pCDNA3.1 expression plasmid (0.25 µg) that harbored the indicated form of CFTR was transfected into HEK 293 cells at 18 hours prior to harvest. CFTR fragment expression was detected by western-blot with the CFTR N-terminal tail antibody (MM13-4). In panel B, the level of respective CFTR fragment was quantitated with a GE Healthcare LAS 4000 and compared to levels detected in the presence of the proteasome inhibitor Bortezomib (10 µM), which was added to cultures 4 hours prior to harvest. This was done because CFTR fragments 370X, 373X, and 375X have a short-half life and do not accumulate to detectable levels unless they are stabilized by folding modulators or inhibition of the proteasome. In C, changes in CFTR signals over the course of a 2-hour chase reaction were normalized to the CFTR signal at T=0.

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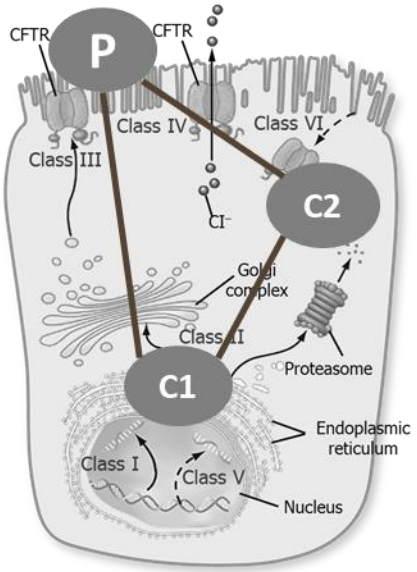
Figures



400 Variants are interpreted on //CFTR2.org

- CF-causing: 336
- Variants of varying clinical consequence: 35
- Non CF-causing: 20
- Variants of unknown significance: 9

Figure 1

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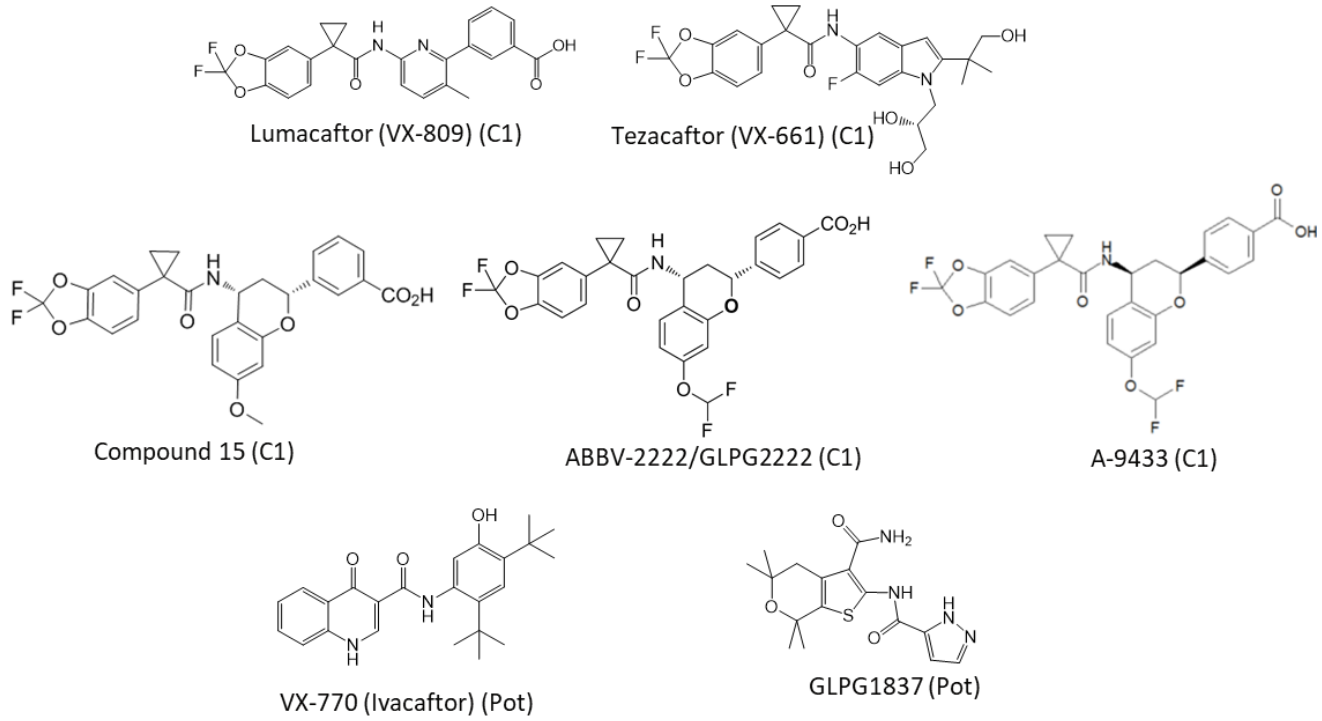

Figure 2

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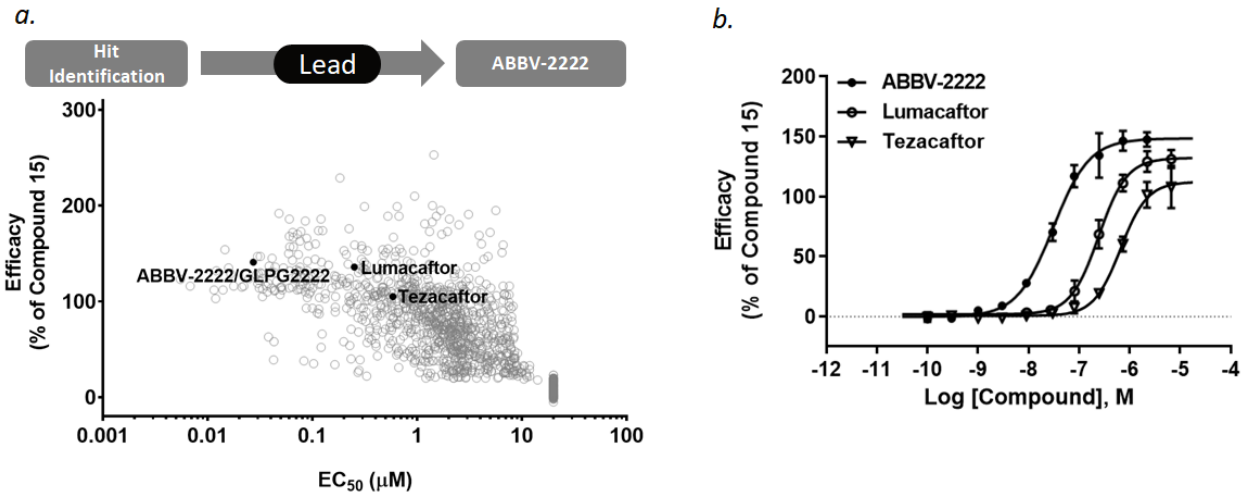

Figure 3

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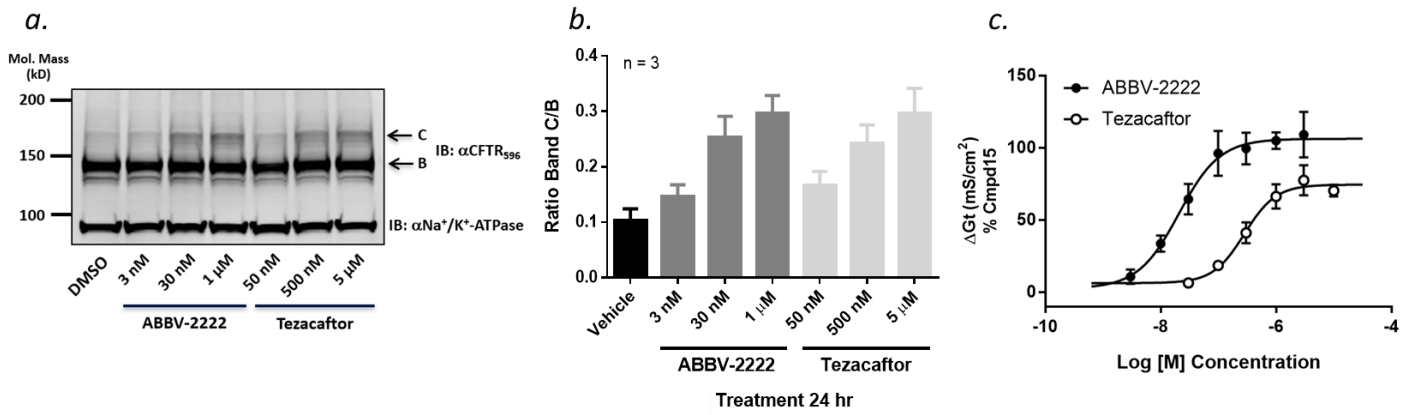

Figure 4

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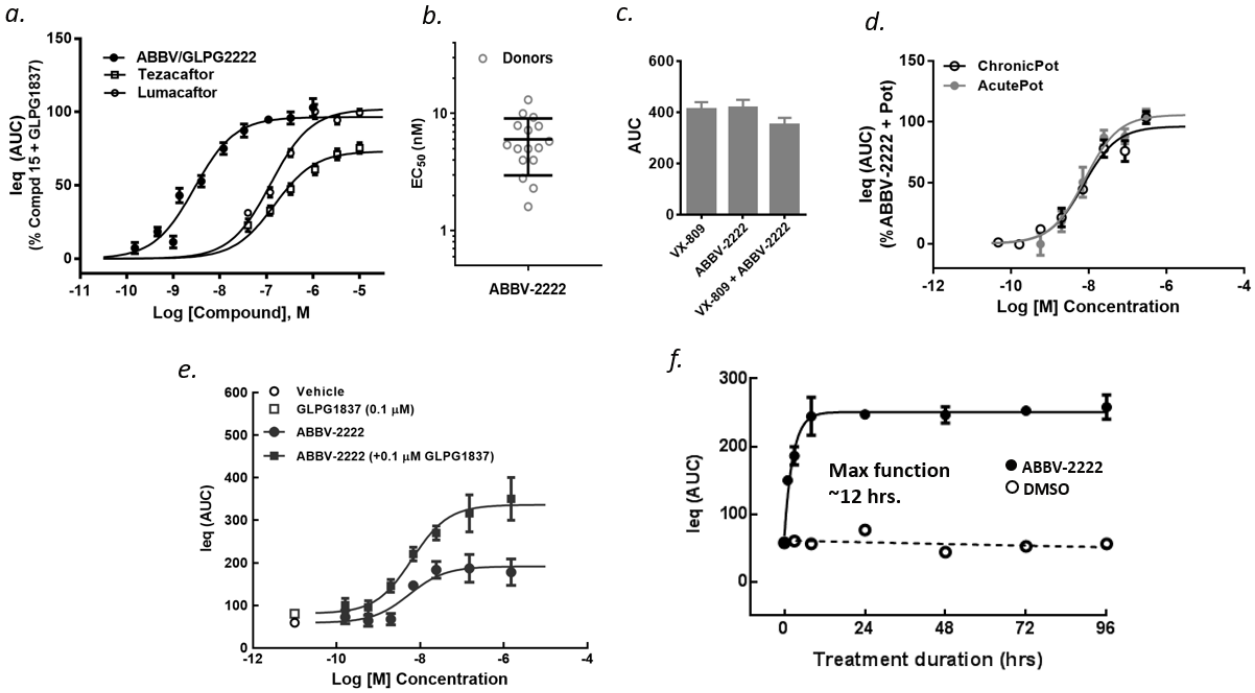

Figure 5

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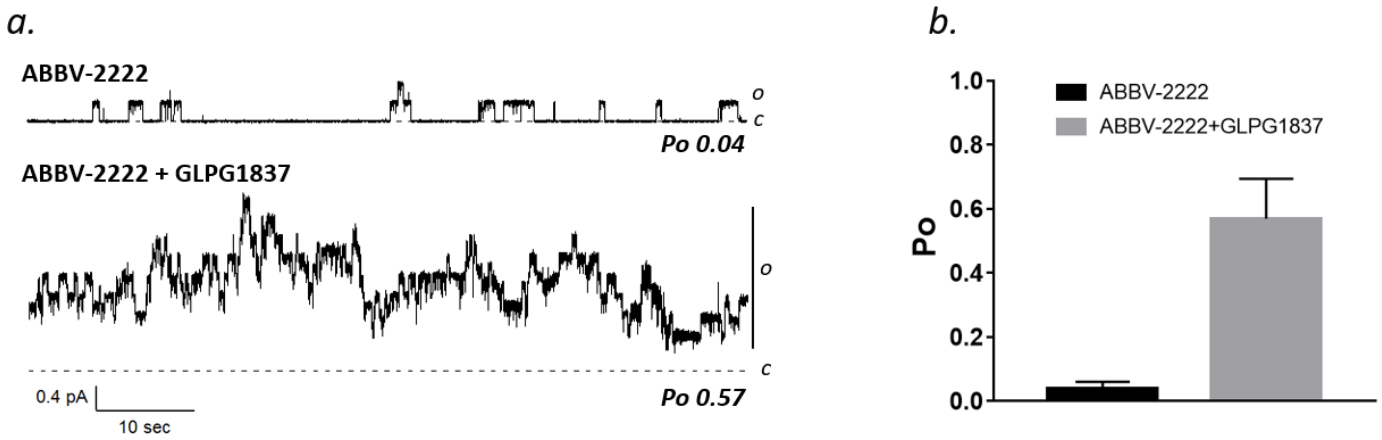

Figure 6

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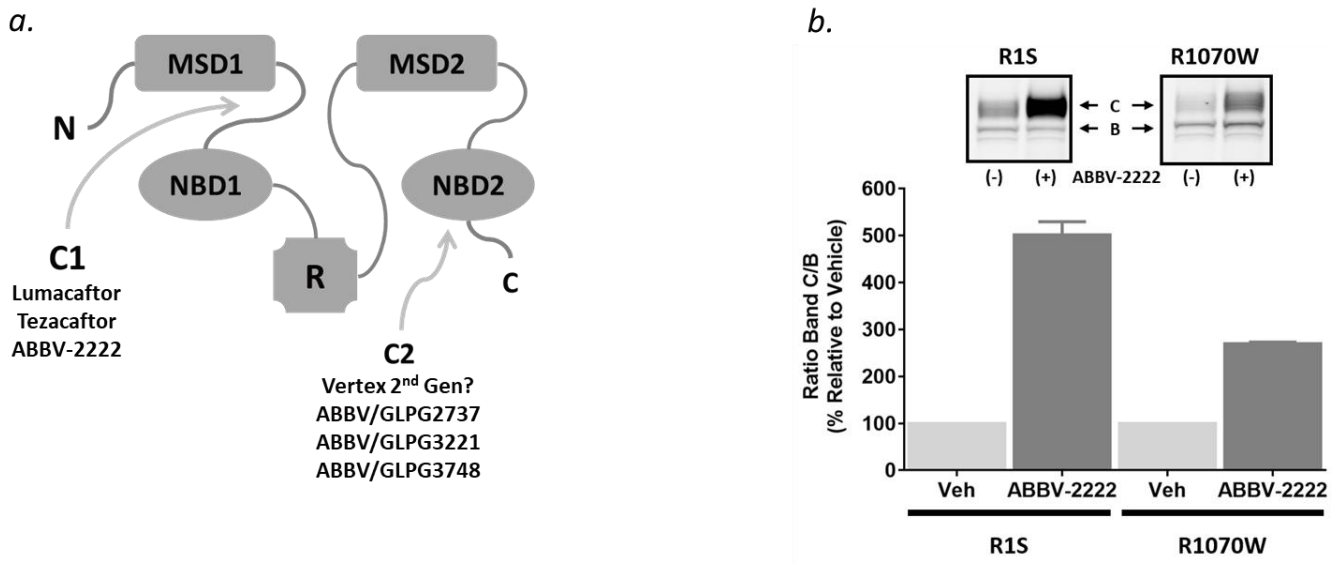

Figure 7

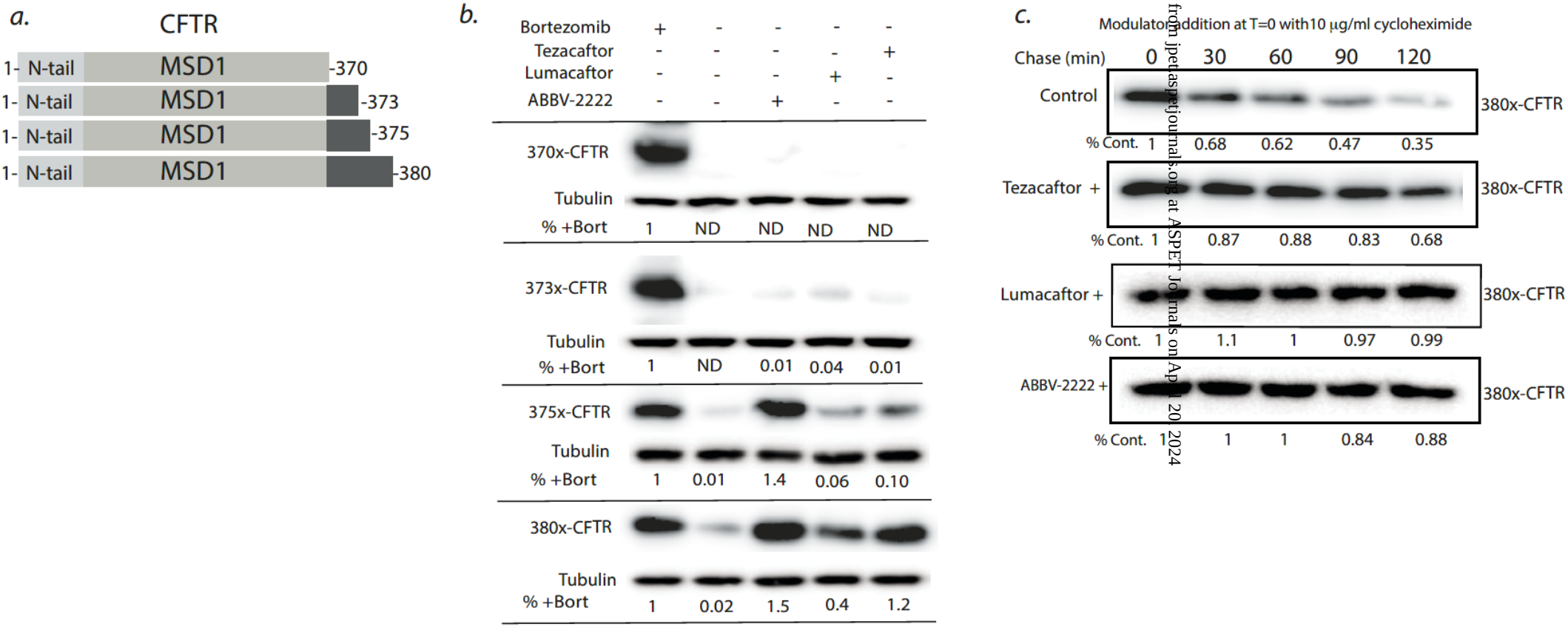

Figure 8