



Corrector-mediated rescue of misprocessed CFTR mutants can be reduced by the P-glycoprotein drug pump

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ABSTRACT

The most common cause of cystic fibrosis is deletion of Phe508 in the first nucleotide-binding domain (NBD) of the CFTR chloride channel, which inhibits protein folding. Δ F508 CFTR can be rescued by indirect approaches such as low temperature but the protein is unstable. Here, we tested our predictions that (1) other CFTR mutants such V232D and H1085R were more stable at the cell surface than Δ F508 CFTR after low temperature rescue and (2) the advantages of rescue with specific correctors (pharmacological chaperones) are that they may stabilize Δ F508 CFTR and increase the effectiveness of the correctors by bypassing drug pumps such as P-glycoprotein (P-gp) (increased bioavailability). It was found that the stability of mutants V232D and H1085R after low-temperature (30 °C) rescue was about 10-fold higher than Δ F508 CFTR. We show that the corrector, 4,5,7-trimethyl-N-phenylquinolin-2-amine (5a), could stabilize Δ F508 CFTR at the cell surface. Unlike most correctors, corrector 5a showed specificity for CFTR as it did not rescue the G268V P-gp processing mutant nor stimulate the ATPase activity of wild-type P-gp. By contrast, corrector KM11060 was a P-gp substrate as it stimulated P-gp ATPase activity and rescued the G268V mutant. Expression of wild-type P-gp reduced the effectiveness of CFTR rescue by corrector KM11060 by about 5-fold. The results underlie the importance of selecting correctors that are specific for CFTR because their efficiency can be reduced by drug pumps such as P-gp.

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1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) is a cAMP-regulated chloride channel that is located on the apical surface of epithelial cells that line lung airways and ducts of various glands (reviewed in [1]). Its physiological role is to regulate salt secretion and reabsorption to maintain normal salt and water homeostasis in epithelial tissues [2].

Cystic fibrosis (CF) is a genetic disease caused by mutations in the CFTR gene that impair synthesis and trafficking of the protein or cause reduced chloride channel activity [3]. The most common defect is deletion of Phe508 (Δ F508 CFTR) in the first nucleotide-binding domain (NBD1). The Δ F508 CFTR protein undergoes rapid degradation in the endoplasmic reticulum (ER) [4] and at the cell

surface because it is defective in folding [5]. The lack of chloride channel activity in CF patients due to defects in CFTR leads to mucosal obstruction of a variety of ducts within organs such as the pancreas, liver, salivary glands, sweat glands and lungs [6]. The main cause of morbidity in CF patients is the presence of thick tenacious secretions that obstruct distal airways and submucosal glands in the lung. These patients have recurrent bouts of lung infections that result in a decline in respiratory function and eventual lung failure.

A potential treatment for CF would be to promote folding of Δ F508 CFTR to increase the amount of protein delivered to the cell surface (reviewed in [7]). Indirect approaches such as low temperature rescue [8], expression in the presence of 'chemical chaperones' such as glycerol, TMAO, or DMSO [9,10], perturbing CFTR–chaperone interactions with agents such as thapsigargin or miglustat [11,12], or inhibiting ER associated degradation [13], have been shown to yield mature Δ F508 CFTR at the cell surface that retains some functional activity. A potential problem with indirect rescue approaches is that they yield a Δ F508 CFTR molecule at the cell surface that is about 5–10 times less stable than the wild-type protein [14]. We predict that rescue of Δ F508 CFTR with specific compounds will yield a more stable protein at the cell surface and reduce problems caused by altering expression

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; TMD1+2, truncated CFTR mutant containing residues 1–388 (TMD1) and residues 847–1196 (TMD2); TMD, transmembrane domain; NBD, nucleotide-binding domain; P-gp, P-glycoprotein.

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of proteins involved in other cellular metabolic pathways. Specificity is also important to bypass drug pumps such as P-glycoprotein (P-gp). P-gp reduces the bioavailability of hydrophobic drugs by inhibiting absorption from the intestine and mediating their efflux through the liver and kidney [15].

We recently showed that it was possible to specifically promote maturation of Δ F508 CFTR to yield a stable protein at the cell surface using a compound that binds directly to CFTR (benzbromarone) [16]. Benzbromarone rescue differed from most other correctors because rescue was specific, as it did not promote maturation of processing mutants of the structurally similar P-gp drug pump. CFTR and P-gp are predicted to be structurally similar and utilize similar biosynthetic pathways because both are members of the ATP-Binding cassette (ABC) family of proteins that contain two transmembrane (TM) domains (TMDs) (each containing 6 TM segments) and two NBDs [17]. Unfortunately, benzbromarone is unsuitable for treatment of CF because it inhibits CFTR activity by blocking the channel [18]. We predicted that direct binding of specific correctors to a site that does not inhibit channel activity would yield a more stable form of Δ F508 CFTR when compared to rescue with indirect methods such as expression at low temperature. We also predicted that specificity is important because correctors that are also substrates of drug pumps such as P-gp would be less efficacious in rescuing misprocessed CFTR mutants. We tested these predictions by using correctors that do not inhibit CFTR activity. We show that rescue of Δ F508 CFTR with specific correctors resulted in a more stable protein and mutations such as V232D and H1085R are different from Δ F508 because they do not destabilize the rescued form of mature CFTR. We also show that P-gp could indeed reduce the effectiveness of a non-specific corrector.

2. Materials and methods

2.1. Chemicals

Correctors N-phenyl-4-(4-vinylphenyl)thiazole-2-amine (2b), 2-(6-methoxy-4-methylquinazolin-2-yl)aminopyrimidin-4(1H)-one (3d), N-(2'-(2-methoxyphenylamino)-4-methyl-5,5'-bithiazol-2-yl)benzamide (4d), N-(2-(5-chloro-2-methoxyphenylamino-4'-methyl-4,5'-bithiazol-2'-yl)pivalamide (15Jf), 2-[1-[4-(4-chlorobenzenesulfonyl)-piperazin-1-yl]-ethyl]-4-piperidin-1-yl-quinazoline (VX-640), 7-chloro-4-(4-(4-chlorophenylsulfonyl)piperazin-1-yl)quinoline (KM11060), 4,5,7-trimethyl-N-phenylquinolin-2-amine (5a), and N-(4-bromophenyl)-4-methylquinolin-2-amine (5c) were obtained from Cystic Fibrosis Foundation Therapeutics, Inc. and Dr. Robert Bridges (Rosalind Franklin University, Chicago, IL, USA). N-(5-Methyl-1,3-thiazol-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-2-carboxamide (EPX-1) was obtained from ChemBridge Corporation (San Diego, CA). N-(6-Ethoxybenzothiazol-2-yl)-2-[5-[(4-hydroxyphenyl)methyl]-4-methylimidazol-2-yl]thioacetamide (EPX-2) was obtained from TimTec LLC (Newark, DE). 5-(4-Nitrophenyl)-2-furaldehyde 2-phenylhydrazone (RDR1) was obtained from MayBridge Ltd (Cornwall, United Kingdom) and 3-(6-[[1-(2,2-difluorobenzoyl)oxy]dioxol-5-yl]-cyclopropanecarbonyl]-amino)-3-methylpyridin-2-yl)-benzoic acid (VX-809) was obtained from Selleck Chemicals LLC (Houston, TX). Dulbecco's modified Eagle's media (DMEM) and calf serum were obtained from Wisent Inc. (St. Bruno, Quebec). Benzbromarone (3-(3,5-dibromo-4-hydroxybenzoyl)-2-ethylbenzofuran), sheep brain phosphatidylethanolamine (Type II-S, Sigma), rhodamine B and Triton X-100 were obtained from Sigma-Aldrich (Oakville, Ontario). Biotin-LC-hydrazide was from Pierce (Rockford, IL). Protease inhibitors Cocktail Set III was from Calbiochem (La Jolla, CA). Nickel-NTA spin columns for purification of histidine-tagged P-gp were purchased from Qiagen Inc.

(Mississauga, Ontario). Monoclonal antibody against GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody A52 and rabbit polyclonal antibody against CFTR were generated as described previously [19,20]. Streptavidin-conjugated horseradish peroxidase was from KPL Inc. (Gaithersburg, MD). Chemiluminescence substrate (Luminata Crescendo) was from Millipore Corporation (Etobicoke, Ontario). [³⁵S]Translabel was obtained from MP Biochemicals (Solon, OH).

2.2. Construction and expression of mutants

Mutations were introduced into CFTR or P-gp cDNAs containing an A52 epitope tag (located at the C-terminal ends) by site-directed mutagenesis as described by Kunkel [21]. The CFTR truncation mutant lacking the NBDs (TMD1+2) contained residues 1–388 (TMD1) linked to residues 847–1196 (TMD2). The mutant CFTRs or P-gps were transiently expressed in HEK 293 cells as described previously [22]. HEK 293 cells were transfected with the cDNAs and the medium was changed 4 h later to fresh medium (Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum) containing various correctors (20 μ M). Many correctors have been shown to rescue Δ F508 CFTR at a concentration of 20 μ M [23–25]. Cells were harvested 18 h after the change in medium. Whole cell extracts of cells (from about 50,000 cells) expressing A52-tagged CFTRs or P-gps were subjected to immunoblot analysis using 6.5% (w/v) acrylamide gels and monoclonal antibody A52. An equivalent amount of the sample was loaded onto 10% (v/v) SDS-PAGE gels and subjected to immunoblot analysis with a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GADPH) (internal control).

To test the effects of correctors or processing mutations on the stability of mature CFTR, the mutants were expressed at low temperature (30 °C) for 18 h in the presence or absence of correctors to promote maturation of the protein. Protein synthesis was then stopped by addition of medium containing 0.5 mg/ml cycloheximide. The cells were then incubated at 37 °C for various time periods (0–32 h). Whole cell extracts were subjected to immunoblot analysis as described above.

To test the effect of P-gp on rescue of CFTR, cells were cotransfected with the H1085R CFTR processing mutant plus wild-type P-gp or an inactive P-gp mutant containing mutations to the catalytic carboxylate residues (E556Q/E1201Q) [26]. The cells were incubated for 24 h at 37 °C to allow for expression of P-gp. The cells were then incubated for 24 h in the presence of various concentrations of the corrector KM11060 [27]. Whole cell extracts were subjected to immunoblot analysis as described above.

2.3. Pulse-chase and cell surface labeling

Baby hamster kidney (BHK) cells stably expressing mutant Δ F508 CFTR were grown in DMEM medium containing 10% (v/v) calf serum for 24 h at 30 °C in the absence or presence of 20 μ M 4,5,7-trimethyl-N-phenylquinolin-2-amine (5a) to promote delivery of mature CFTR to the cell surface. The next day protein synthesis was stopped by addition of 0.5 mg/ml cycloheximide and the cells were incubated at 37 °C for 0–32 h. The cells were washed four times with phosphate buffered saline (pH 7.4) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBSCM) and then treated in the dark with PBSCM buffer containing 10 mM NaIO₄ for 30 min at 4 °C. The cells were then washed four times with PBSCM buffer and treated with sodium acetate buffer (100 mM sodium acetate buffer, pH 5.5, 0.1 mM CaCl₂, 1 mM MgCl₂) containing 2 mM biotin-LC-hydrazide for 30 min at 20 °C. The cells were then washed twice with sodium acetate buffer and solubilized with Tris-buffered saline (100 mM Tris-HCl, pH 7.4 and 150 mM NaCl) containing 1% (w/v) Triton X-100 and protease inhibitors.

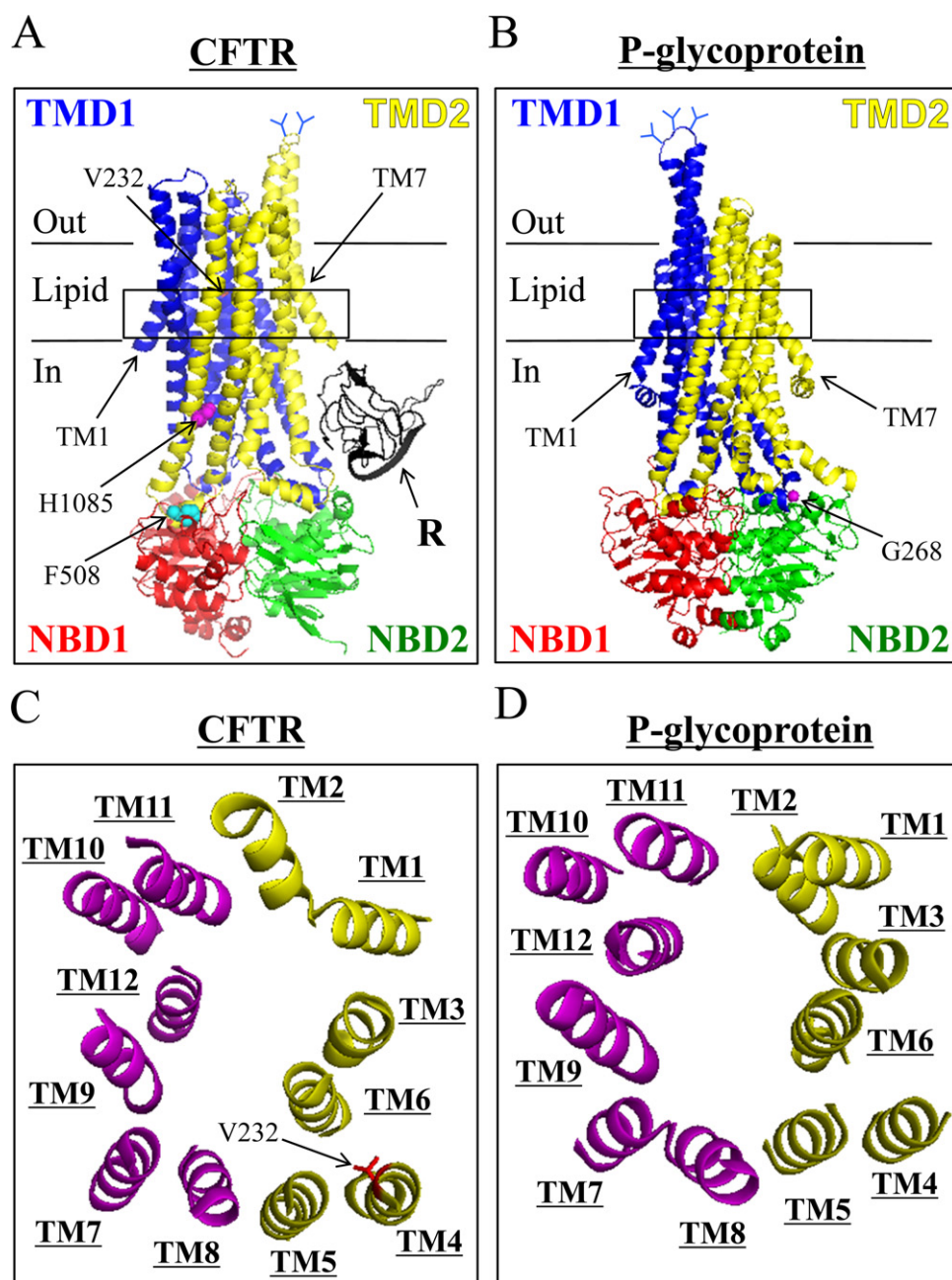


Fig. 1. Homology models of CFTR and P-glycoprotein are similar. Predicted structures of CFTR (A) [17] or P-gp (B) [59] and viewed using Pymol [60]. The locations of the nucleotide-binding domains (NBD1 (red); NBD2 (green)) and transmembrane domains (TMD1 (blue); TMD2 (yellow)) and the R domain (grey) of CFTR are shown. Each TMD contains six TM segments and the pores are predicted to be located at the TMD1–TMD2 interfaces. The branched lines (light blue) represent the glycosylation sites. The glycosylation sites are located in the first extracellular loop of TMD1 in P-gp and the first extracellular loop of TMD2 in CFTR. Phe508 (F508) in CFTR is located in NBD1 adjacent to cytoplasmic loop 4 (blue) in TMD2 that connects the cytoplasmic extensions of transmembrane segments 10 and 11. The locations of residues V232 (TMD1) and H1085 (TMD2) in CFTR and G268 in P-gp are indicated. The models are consistent with cross-linking results of CFTR [61] or P-gp [62] that show that the NBDs of CFTR interact in a head-to-tail configuration. Overhead views of packing of the transmembrane segments of the proteins in the inner leaflet of the lipid bilayer (boxed regions) are shown in C (CFTR) and D (P-gp). The location of V232 in TM4 of CFTR is indicated.

CFTR was immunoprecipitated with monoclonal antibody A52, subjected to SDS-PAGE on 6.5% gels and biotinylated CFTR was detected with streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence. Pulse-chase with [35 S]Translabel was performed on BHK cells stably expressing Δ F508 CFTR in the presence or absence of 20 μ M 5a as described previously [28].

2.4. Purification of P-gp and assay of ATPase activity

Histidine-tagged P-gp was isolated by nickel-chelate chromatography as described previously [29]. A sample of the isolated

histidine-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine that had been washed and suspended in TBS (Tris-buffered saline: 10 mM Tris-HCl, pH. 7.4 and 150 mM NaCl). The sample was sonicated and ATPase activity measured in the absence of drug substrate or in the presence of 0.3 mM corrector or the drug substrate rhodamine B. The samples were incubated for 30 min at 37 °C and the amount of inorganic phosphate released was determined [30]. A higher concentration of correctors was used in the ATPase assays (0.3 mM) compared to the rescue experiments (20 μ M) because the ATPase assays are less sensitive due to the presence of high amounts of lipid and detergent.

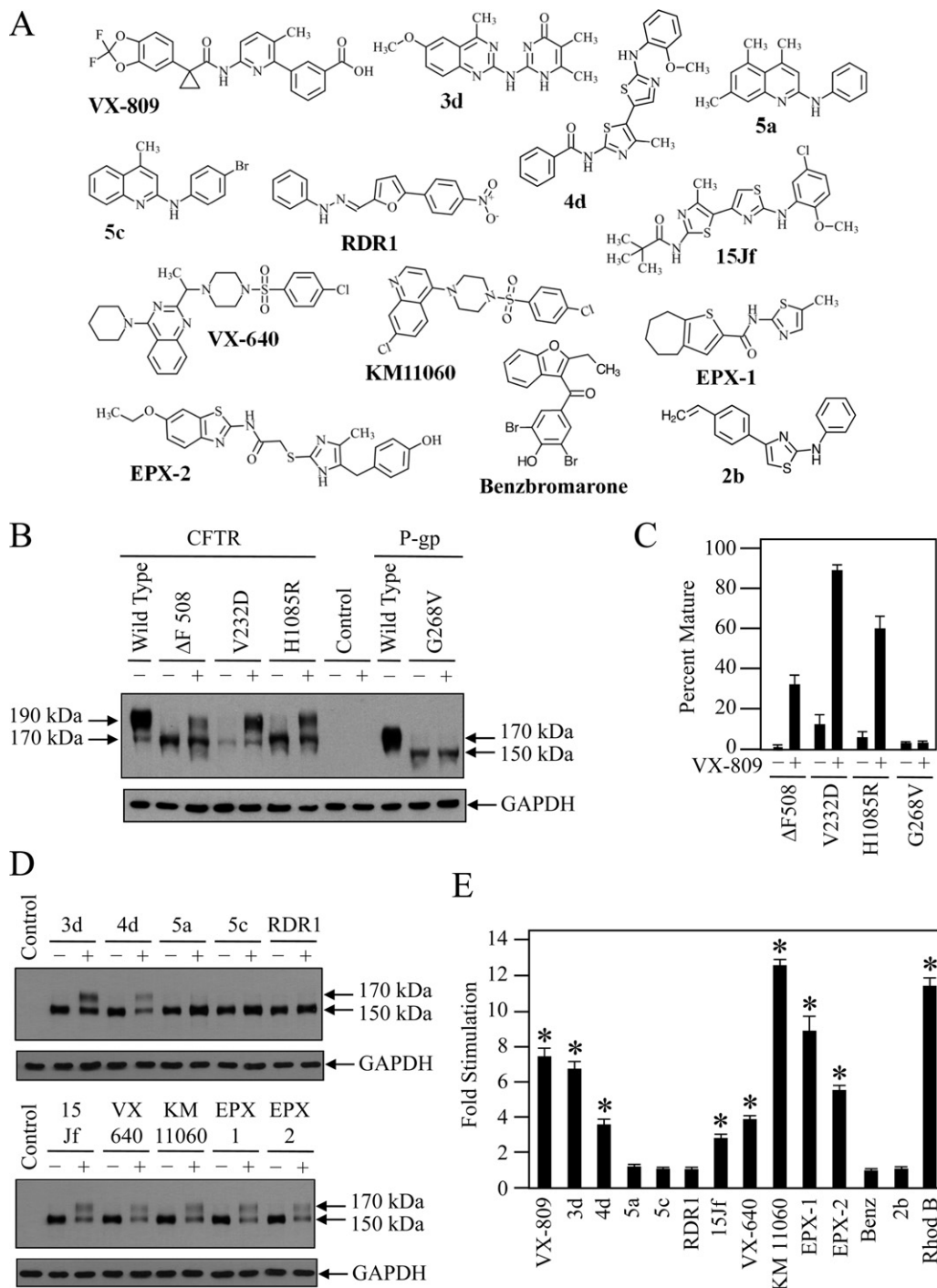


Fig. 2. Effect of correctors on maturation of G268V P-gp. (A) Structure of correctors. (B) Samples of cells expressing Δ F508, V232D or H1085R CFTR mutants or P-gp mutant G268V and grown in the absence (–) or presence (+) of 20 μ M VX-809 for 18 h were subjected to immunoblot analysis. Cells transfected with wild-type CFTR, wild-type P-gp or vector alone (Control) were included as controls. The amount of mature CFTR or P-gp (Percent Mature) relative to total (mature plus immature) is shown in (C). Each value is the mean ($n = 3$) \pm SD. (D) Rescue of P-gp mutant G268V after expression for 18 h in the absence (–) or presence (+) of 20 μ M of the indicated corrector. The positions of the mature CFTR (190 kDa), mature P-gp (170 kDa), immature CFTR (170 kDa) and immature P-gp (150 kDa) are indicated. (E) Stimulation of purified wild-type P-gp ATPase activity was assayed in the presence of correctors. The fold-stimulation is the ratio of ATPase activity in the presence of 0.3 mM corrector, benzbromarone (Benz), or P-gp substrate rhodamine B (Rhod B) to the activity in the absence of corrector. The results are the mean of three measurements \pm SD. An asterisk indicates significant difference ($P < 0.05$) when compared to that without corrector.

2.5. Data analysis

The amount of product in each lane in SDS–PAGE gels was determined by scanning the gel lanes followed by analysis with the NIH Image program (available at <http://rsb.info.nih.gov/nih-image>) and an Apple computer. The results were expressed as the average of triplicate experiments \pm standard deviation (SD). Statistical significance ($P < 0.05$) was determined using the Student's two-tailed *t* test.

3. Results

3.1. Effect of CFTR correctors on maturation and activity of P-gp

We predicted that specific correctors would have similar effects as benzbromarone in promoting maturation of Δ F508 CFTR to yield a more stable protein. The first step was to identify specific correctors. The P-gp drug pump was selected to test the specificity

Table 1
Effect of correctors on maturation of G268V P-gp and on P-gp ATPase activity.

Corrector	Maturation G268V P-gp ^a	P-gp ATPase activity ^b
VX-809	–	+
3d	+	+
4d	+	+
5a	–	–
5c	–	–
RDR1	–	–
15Jf	+	+
VX-640	+	+
KM11060	+	+
EPX-1	+	+
EPX-2	+	+
Benzbromarone	–	–
2b	–	–

^a (+) increased or (–) no effect on maturation when compared to a sample without corrector.

^b (+) increased or (–) no effect on P-gp ATPase activity when compared to a sample without corrector.

of various correctors for two reasons. First, P-gp and CFTR are predicted to be structurally similar glycosylated ABC proteins (see Fig. 1) that likely follow similar protein folding and trafficking pathways. For example, processing mutations in either P-gp or CFTR trap the proteins in the ER as partially folded biosynthetic intermediates with incomplete domain interactions [22] and packing of the TM segments [20,31]. Second, P-gp would reduce the bioavailability of correctors if they are substrates of this drug pump and substrates of P-gp can increase expression by promoting folding of the protein [32].

The P-gp processing mutant G268V was used to test the specificity of various correctors (structures are shown in Fig. 2A) identified by Pedemonte et al. (corr-3d, corr-4d, corr-5a, corr-5c) [33], van Goor et al. (VX-640) [34] and VX-809 [25], Carlile et al. (sildenafil analog KM11060) [27,35], Sampson et al. (RDR1) [24], and Kalid et al. (e.g. EPX-106817 (EPX-1), EPX-107860 (EPX-2) predicted to bind at domain interfaces in a structure-based virtual screening approach) [23]. P-gp mutant G268V was selected because it was previously used to test the specificity of VX-325 [36] and it shows properties that are characteristic of all P-gp processing mutants [37]. Some common properties of P-gp processing mutations are that they disrupt domain-domain interactions and packing of the TM segments. These defects could be repaired by expressing the mutants in the presence of drug substrates.

We also tested whether correctors would promote maturation of CFTR mutants Δ F508, V232D and H1085R in HEK 293 cells in parallel because it has been reported that CFTR rescue depends on the cell system used [38–41]. Mutants V232D (TMD1) and H1085R (TMD2) were included because they are processing mutations located in different domains of CFTR (Fig. 1A) and both yield active proteins after rescue [16,42,43]. An example of rescue with VX-809 is shown in Fig. 2B and C. VX-809 promoted maturation of all three CFTR processing mutants. VX-809 however, did not promote maturation of G286V P-gp. Similarly, only some of the correctors promoted maturation of G268V P-gp (Fig. 2D). Correctors 3d, 4d, 15Jf, VX-640, KM11060, EPX-1 and EPX-2 promoted maturation of the P-gp mutant to yield the (mature) 170 kDa protein. Correctors 5a, 5c and RDR1 showed specificity because they did not promote rescue of G268V.

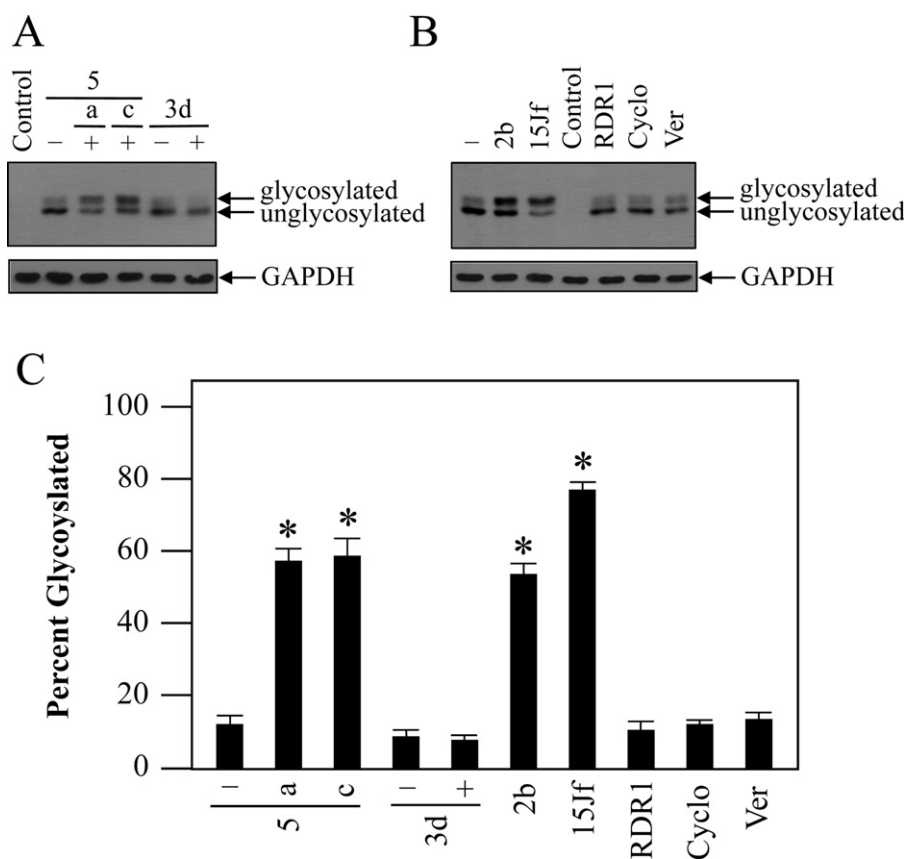


Fig. 3. Glycosylation of CFTR truncation mutant TMD1+2. CFTR TMD1+2 was expressed in the absence (–) or presence (+) of correctors 5a, 5c, 3d (A), 2b, 15Jf, RDR1, the P-gp substrates cyclosporine A (Cyclo), verapamil (Ver) (B) or cells not expressing TMD1+2 (Control) (A). The positions of glycosylated and unglycosylated TMD1+2 are shown and the relative level of glycosylated protein was determined (C). The results are the mean of three experiments \pm SD. An asterisk indicates significant difference ($P < 0.05$) when compared to that with no corrector.

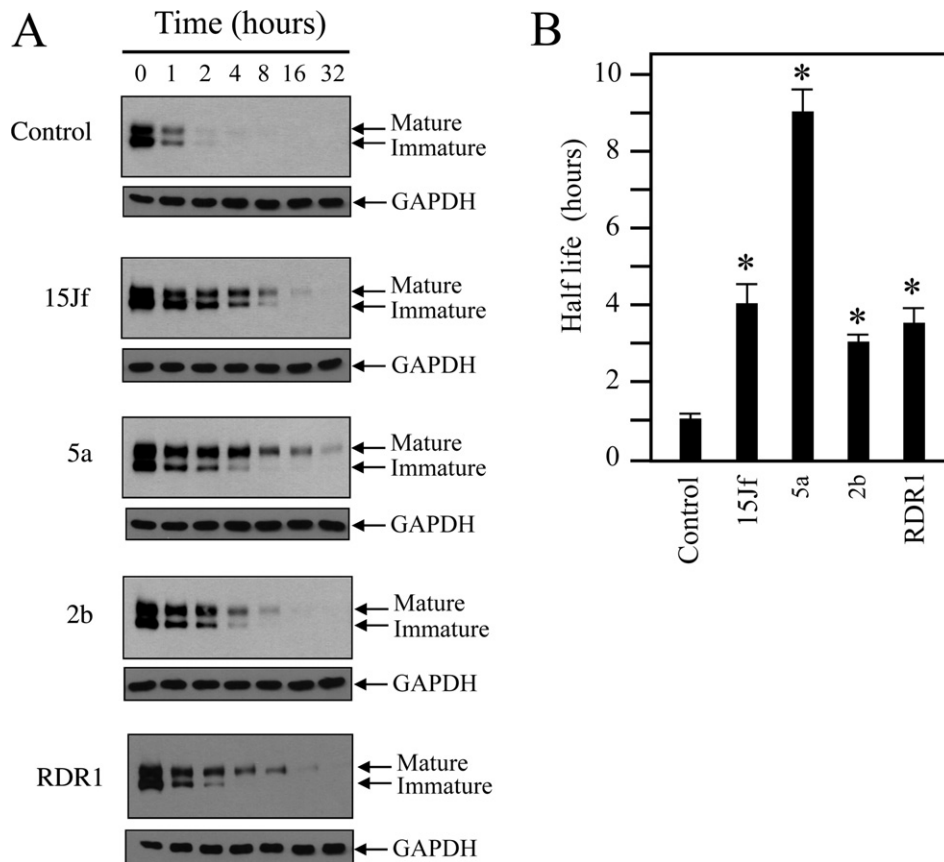


Fig. 4. Effect of correctors on Δ F508 CFTR turnover. Degradation of Δ F508 CFTR at 37 °C was determined in the absence (Control) or presence of 20 μ M of the indicated correctors (A). Cells were first incubated at 30 °C to promote maturation of Δ F508 CFTR. Protein synthesis was stopped by addition of 0.5 mg/ml cycloheximide. The positions of mature and immature CFTR and GAPDH (loading control) in the immunoblots are indicated. The amount of mature CFTR at each time point was quantitated and expressed relative to time 0 (B). The results are the mean of three experiments \pm SD. An asterisk indicates significant difference ($P < 0.05$) when compared to that with no corrector.

Correctors that rescued P-gp mutant G268V (3d, 4d, VX-640, KM11060, 15Jf, EPX-1, EPX-2) may be acting as pharmacological chaperones that directly interact with the protein to promote maturation. To test for direct interaction of P-gp with the correctors, we assayed for stimulation of ATPase activity using purified wild-type P-gp. Corrector 2b was included as a negative control, as we previously showed that it did not stimulate P-gp ATPase activity [44]. The P-gp substrate rhodamine B [45,46] was used as a positive control. Benzbromarone was tested as we previously showed that it did not promote maturation of a P-gp processing mutant [16]. It was found that all the correctors that rescued P-gp mutant G268V (3d, 4d, VX-640, KM11060, 15Jf, EPX-1, EPX-2) stimulated ATPase activity. Most correctors that did not rescue G268V P-gp (5a, 5c, RDR1, 2b) did not activate ATPase activity (summarized in Table 1). The results suggest that

correctors rescued mutant G268V through direct interactions with P-gp.

One exception was VX-809. It stimulated P-gp ATPase activity about 7-fold, but we also found that it did not promote maturation of G268V P-gp (Fig. 2B) or other P-gp processing mutants (data not shown). VX-809 resembles P-gp substrates such as colchicine and doxorubicin that stimulate ATPase activity but do not rescue P-gp processing mutants (data not shown).

3.2. Effect of CFTR correctors on glycosylation and stability of CFTR

The quinoline compounds (5a, 5c) and RDR1 were similar to benzbromarone [16] because they did not promote maturation of a P-gp processing mutant. It was previously found that the mechanism of benzbromarone rescue was to promote folding of the transmembrane domains in the ER because it enhanced core-glycosylation of a CFTR truncation mutant lacking the NBDs [16]. We predicted that the quinoline compounds (5a and 5c) would also promote folding of a mutant lacking the NBDs and that corrector RDR1 would not because it was shown to interact with NBD1 of CFTR [24]. To test if the quinoline compounds or RDR1 could influence folding of a CFTR mutant lacking the NBDs (TMD1+2; shows only about 10% core-glycosylation efficiency), the TMD1+2 mutant was expressed in the presence or absence of 20 μ M 5a, 5c or RDR1.

Several control compounds were included. To test if other hydrophobic compounds could non-specifically alter the ER environment to promote glycosylation of CFTR TMD1+2, the mutant was expressed in the presence of 20 μ M verapamil or cyclosporine A. Verapamil and cyclosporine A have previously

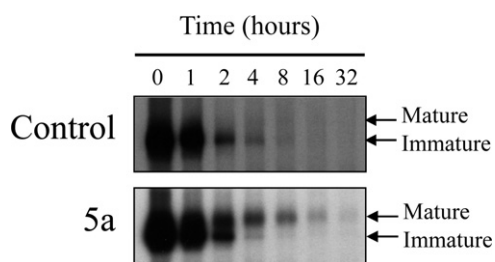


Fig. 5. Effect of 5a on maturation of Δ F508 CFTR. Pulse-chase assays of Δ F508 CFTR were performed in the absence (Control) or presence of 20 μ M 5a. The positions of mature and immature forms of CFTR are indicated.

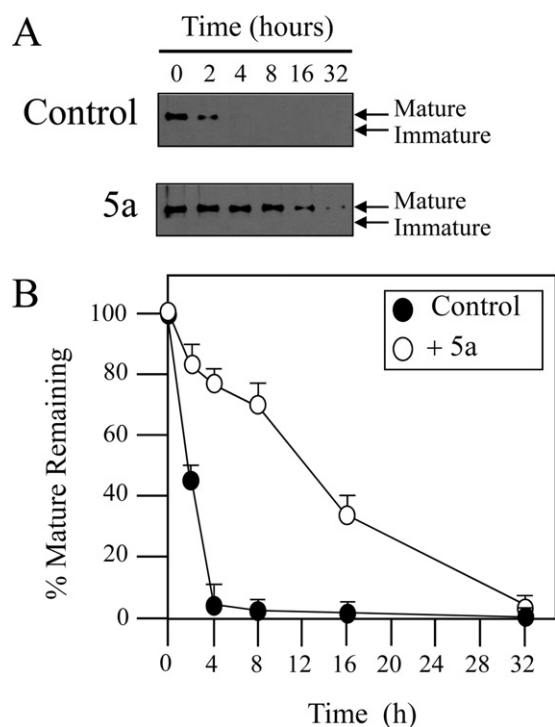


Fig. 6. Effect of 5a on turnover of Δ F508 CFTR at the cell surface. Cell surface labeling was performed on cells expressing Δ F508 CFTR in the presence or absence (Plain) of 20 μ M 5a (A). Cells were first incubated for 24 h at 30 °C in the absence (Control) or presence of 5a to promote maturation of the protein. Protein synthesis was stopped by incubating the cells in 0.5 mg/ml cycloheximide and cell surface labeling was performed after the indicated times at 37 °C. The amount of labeled CFTR at each time point was quantitated and expressed relative to time 0 (B). The results are the average of three experiments \pm SD.

been shown to promote folding of a P-gp mutant lacking the NBDs (P-gp TMD1+2) in the ER [47]. Corrector 15Jf was tested because it is a high-affinity bithiazole derivative [48]. Bithiazole derivatives have previously been shown to promote maturation of CFTR processing mutants through interactions in the ER [42]. In addition, corrector 3d was included because it had previously been reported

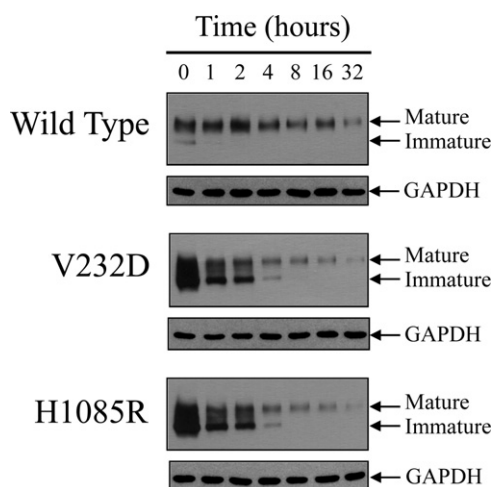


Fig. 7. Stability of other CFTR mutants. Degradation of wild-type, V232D, and H1085R CFTRs was monitored over time at 37 °C. The cells were first incubated at 30 °C to promote maturation of CFTR. Protein synthesis was then stopped by addition of 0.5 mg/ml cycloheximide and whole cell extracts were subjected to immunoblot analysis after the indicated times at 37 °C. The positions of mature and immature CFTR and GAPDH (loading control) in the immunoblots are indicated.

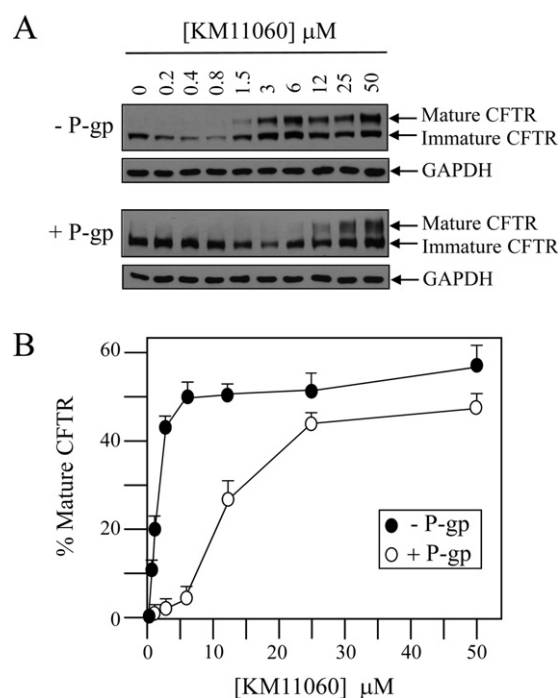


Fig. 8. Effect of wild-type P-gp expression (+P-gp) on rescue of CFTR mutant H1085R with corrector KM11060. (A) The positions of mature and immature CFTR and the GAPDH loading control in the immunoblots of whole cell extracts from cells incubated with the indicated concentration of corrector are shown. HEK 293 cells were co-transfected with an inactive P-gp mutant (– P-gp) or with wild-type P-gp (+ P-gp). (B) Relative amount of mature to total CFTR (mature plus immature) at different concentrations of KM11060 when cells were expressed in the absence (–) or presence (+) of wild-type P-gp. Each value is the mean of three experiments \pm SD.

that type 3 correctors did not promote maturation of Δ F508 CFTR in the ER [33] while 2b was included because we previously showed it was specific for rescue of CFTR [44].

It was found that 5a, 5c, 2b, and 15Jf enhanced glycosylation of CFTR TMD1+2 (Fig. 3A and B). The most effective corrector was 15Jf as 77 \pm 5% of TMD1+2 was glycosylated after expression in the presence of this corrector (Fig. 3C). About 50–60% of TMD1+2 was glycosylated after expression in the presence of correctors 5a, 5c, or 2b. The increase in TMD1+2 glycosylation did not appear to be caused by nonspecific perturbations of the ER by hydrophobic compounds because no increase in glycosylation was observed in the presence of the hydrophobic P-gp substrates verapamil or cyclosporine A (Fig. 3B). No increase in glycosylation (<1.5-fold increase) was observed when TMD1+2 was expressed in the presence of correctors 3d (Fig. 3A) or RDR1 (Fig. 3B). These results suggest that the mechanisms of CFTR correctors like 5a, 5c, 2b, and 15Jf resembled benzobromarone such that they altered folding of CFTR while it was being synthesized in the ER.

One mechanism of benzobromarone rescue of Δ F508 CFTR was that it enhanced stability of the mutant [16]. To test if correctors that promoted glycosylation of TMD1+2 also promoted stability of Δ F508 CFTR, turnover of the full-length protein was assayed after rescue with the correctors 15Jf, 5a, and 2b. Corrector RDR1 was also included because it showed specificity, as it did not promote maturation of the P-gp processing mutant (Fig. 2D). The mutant was expressed in the presence or absence of 20 μ M of the various correctors for 18 h at 30 °C to promote maturation of the protein. Protein synthesis was then stopped by addition of 0.5 mg/ml cycloheximide. After 30 min of incubation in the presence of cycloheximide the cells were then placed in an incubator at 37 °C for various time periods (0–32 h). Whole cell extracts were

subjected to immunoblot analysis to monitor turnover of the protein.

In the absence of correctors, turnover of both the mature and immature proteins was rapid with half-lives of 1.0 ± 0.3 and 0.5 ± 0.1 h, respectively (Fig. 4). Correctors 15Jf, 5a, 2b, and RDR1 increased the half-life of the mature protein by 3- to 9-fold (4.0 ± 0.7 , 9.1 ± 1.0 , 3.2 ± 0.2 , and 1.0 , 3.7 ± 0.4 h, respectively).

Corrector 5a was selected for further study in a pulse-chase assay since it was specific (Fig. 2D) and caused the largest increase in stability of mature $\Delta F508$ CFTR (Fig. 4). A pulse-chase assay was performed on BHK cells expressing $\Delta F508$ CFTR in the presence or absence of $20 \mu\text{M}$ 5a. It was observed that 5a promoted maturation of $\Delta F508$ CFTR to yield a product with a half-life of about 9 h (Fig. 5). These results show that $\Delta F508$ CFTR was stabilized whether rescue with 5a was performed at 30°C or 37°C .

Turnover of $\Delta F508$ CFTR from the cell surface was then monitored by cell surface labeling. BHK cells expressing $\Delta F508$ CFTR were incubated at 30°C for 18 h in the presence or absence of $20 \mu\text{M}$ 5a to promote maturation of the protein. Protein synthesis was then stopped by addition of 0.5 mg/ml cycloheximide and the cells were placed at 37°C . After various times, CFTR at the cell surface was identified by biotinylation (Fig. 6). In the absence of 5a, $\Delta F508$ CFTR had a half-life of 1.7 ± 0.4 h. Expression in the presence of 5a however, stabilized $\Delta F508$ CFTR at the cell surface as its half-life increased to 12 ± 2 h.

3.3. Effect of other CF mutations on stability of CFTR

The $\Delta F508$ mutation appears to reduce the stability of CFTR because it inhibits folding of NBD1 and as a consequence disrupts NBD1–TMD2 interactions [17,22,49]. We predicted that processing mutations in other domains, however, would not have such severe deleterious effects on the stability of mature CFTR as $\Delta F508$. To test if other CF mutations affect stability of CFTR, mutants V232D (TMD1) and H1085R (TMD2) were selected for study as both are processing mutations that yield active proteins after rescue [16,43]. Accordingly, we examined the stability of mutants V232D and H1085R after low-temperature rescue. Cells expressing wild-type, V232D, or H1085R CFTRs were expressed at low temperature to promote maturation of the protein. Protein synthesis was stopped by addition of cycloheximide, and turnover of the protein was monitored after incubation for 0–32 h at 37°C (Fig. 7). It was observed that the half-lives of the mature forms of V232D and H1085R were at least 10-fold longer (about 14 and 12 h, respectively) than $\Delta F508$ CFTR (about 1 h, Fig. 4). The half-life of wild-type CFTR was about 18 h (Fig. 7).

The V232D TMD1 and H1085R mutations may have less effect on the stability of mature CFTR because they have more localized effects on protein folding in the TMD1 and TMD2 domains, respectively. The $\Delta F508$ mutation can disrupt folding of NBD1 as well as interdomain interactions [49].

3.4. Effect of P-gp expression on rescue of CFTR mutant H1085R

We predicted that the efficiency of CFTR rescue with correctors would be reduced if the correctors were also substrates of drug pumps such as P-gp. Sildenafil is a non-toxic substrate of human P-gp [50]. Stimulation of P-gp ATPase activity by the sildenafil analog KM11060 shows that it also is a P-gp substrate and rescues mutant G268V likely through a drug-rescue mechanism. An advantage of KM11060 over most correctors is that it is relatively nontoxic to cells at concentrations up to at least $100 \mu\text{M}$ [27] so a wide concentration range can be tested in rescue assays. Most other correctors become toxic to HEK 293 at concentrations above about $30 \mu\text{M}$ (unpublished observations). To test if P-gp expression would alter CFTR rescue with KM11060, mutant H1085R was expressed in the presence of

various concentrations of KM11060 in the presence of an inactive P-gp containing mutations to the catalytic carboxylate residues (E556Q/E1201Q) in the nucleotide binding domains [26] or in the presence of wild-type P-gp. CFTR mutant H1085R was tested because it has a higher efficiency of rescue with correctors compared to $\Delta F508$ CFTR [16] and the mature protein is more stable (Fig. 7). It was found that wild-type P-gp expression reduced the potency of KM11060 about 5-fold (Fig. 8). The concentration of KM11060 required for 50% rescue (R_{50}) increased from $2.0 \pm 1.3 \mu\text{M}$ in the presence of inactive of P-gp to $11.0 \pm 2.5 \mu\text{M}$ in the presence of wild-type P-gp. The R_{50} for KM11060 when H1085R CFTR was expressed with no exogenous P-gp was similar to that obtained with inactive P-gp (data not shown) suggesting that expression of P-gp did not adversely affect the cellular folding machinery.

4. Discussion

Correctors that specifically rescue $\Delta F508$ CFTR might cause fewer side effects compared to indirect rescue approaches because they would not alter expression of proteins involved in other metabolic pathways. In this study, we also showed that another potential advantage of specific correctors is that they may show enhanced bioavailability if they are not substrates of the P-gp drug pump. Expression of the P-gp drug pump reduced the effectiveness of corrector KM11060 by several-fold (Fig. 8). Increasing the bioavailability of correctors will be important because CF patients would likely require daily treatments with correctors and rescue of $\Delta F508$ CFTR by most correctors identified to date require relatively high concentrations for rescue (low micromolar concentrations). For example, recent clinical trials [51] of the corrector VX-809 (developed by Vertex Pharmaceuticals) showed that daily doses of 100–200 mg were required to cause a statistically significant reduction in sweat chloride values but no maturation of $\Delta F508$ CFTR could be detected in rectal biopsies. Our observation that VX-809 was a P-gp substrate suggests that drug pumps may be one of the factors that contributed to low rescue of $\Delta F508$ CFTR in patients compared to cell-based systems. It was observed however, that serum levels of VX-809 administered to rats were high enough for in vitro efficacy [25] although it has been noted that human and rodent P-gps show different substrate specificities [52].

We found that the quinolines (5a and 5c) and RDR1 showed specificity for rescue of CFTR (results summarized in Table 1). In a previous study, we also found that the aminoarylthiazole 2b did not promote maturation of P-gp processing mutants [44]. There is evidence that these specific correctors (RDR1, 2b, 5a, and 5c) may act as pharmacological chaperones by directly interacting with CFTR.

RDR1 has been shown to bind to NBD1 using differential scanning calorimetry [24]. This may explain why RDR1 did not promote glycosylation of the CFTR TMD1+2 truncation mutant as observed with the channel blocker benzbromarone [16]. Direct interactions of RDR1 with NBD1 of $\Delta F508$ CFTR appeared to promote stability of the mature protein by about 3-fold (Fig. 4). It was also reported that the potentiator VX-532 stabilized NBD1 at high concentrations (0.1 mM). VX-532 has been predicted to bind at the NBD–TMD interface of CFTR [53]. It also shows specific corrector activity as it causes a modest increase in maturation of CFTR processing mutants [43,44] but not P-gp processing mutants [44].

The other specific correctors (quinolines and 2b) resembled benzbromarone as they increased the efficiency of glycosylation of the truncation mutant lacking the NBDs by about 5-fold (Fig. 3). Corrector 15Jf was the most effective compound as it enhanced glycosylation of TMD1+2 by over 6-fold. Although corrector 15Jf rescued P-gp, it has been reported that bisaminomethylthiazoles show specificity for CFTR because they did not rescue a dopamine receptor (DRD4) processing mutant (M345T) [33]. These observations suggest that 5a, 5c, 2b, and 15Jf were influencing folding of

TMD2 as it contains the glycosylation sites. It is possible that these correctors influenced glycosylation of TMD2 through indirect effects since there is no evidence that they bind directly to this domain. It has been reported that TMD2 is an unstable domain of CFTR that is chaperoned by calnexin [54]. In a study of the insertion characteristics of CFTR TM segments, it was found that the segments in TMD1 were stably inserted but TMD2 contained several unstable TM segments [55]. It has been reported that Corr-4a (another bisaminomethylbithiazole corrector like 15Jf) may stabilize TMD2 [42]. Corr-4a also may promote interactions between TMD1 and TMD2 as it restored folding of a processing mutant containing a charged residue (V232D) within TM4 [43] that is embedded in the lipid bilayer [56]. The V232D mutation is predicted to alter packing of the TM segments [56].

Corrector 2b also promoted glycosylation of CFTR TMD1+2 and was specific for rescue of CFTR [44]. We observed that it promoted stability of mature Δ F508 CFTR (Fig. 4) in agreement with a study by Pedemonte et al. [33]. There is evidence that 2b related arylaminothiazoles can directly bind to CFTR because they correct defective gating in mutants such as Δ F508 CFTR and G551D [57].

There is evidence that 5a related quinolines can also directly bind to CFTR because cyanoquinolines have been identified that have both corrector and potentiator activity [58]. The potentiator activity of cyanoquinolines differed from arylaminothiazoles because they activated chloride conductance of Δ F508 or G551D CFTRs within minutes. Correction of defective Δ F508 CFTR gating with arylaminothiazoles required incubation of cells for several hours [57].

The results of this study show that several classes of correctors show increased specificity for CFTR because they were not substrates of the P-gp drug pump and did not promote maturation of P-gp mutants expected to utilize similar folding and trafficking pathways. One possibility is that the specific correctors acted as pharmacological chaperones that interacted directly with Δ F508 CFTR to yield a more stable protein at the cell surface. It cannot be ruled out, however, that specific correctors may indirectly stabilize Δ F508 CFTR at the cell surface through indirect effects on endocytosis as observed with correctors VX-325 plus 4a [38] or on ubiquitination pathways as observed with corrector 4a [41]. Further development of these classes of compounds to enhance their abilities to promote maturation and enhance stability of Δ F508 CFTR may yield useful candidates for CF drug therapy.

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