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Rescue of ΔF508 and Other Misprocessed CFTR Mutants by a Novel Quinazoline Compound†

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Abstract: Cystic fibrosis (CF) is most commonly caused by deletion of Phe508 in the cystic fibrosis transmembrane conductance regulator protein (ΔF508 CFTR). The misfolded ΔF508 CFTR protein is retained in the endoplasmic reticulum (misprocessed mutant) and is rapidly degraded. Studies on misprocessed mutants of P-glycoprotein (P-gp), a sister protein of CFTR, however, have shown that specific substrates and modulators can act as specific chemical/pharmacological chaperones to rescue the protein. A major goal in CF research is the identification of compounds that can be used at low concentrations to rescue misprocessed CFTR mutants. Here, we show that a novel quinazoline derivative, 4-cyclohexyloxy-2-{1-[4-(4-methoxy-benzenesulfonyl)piperazin-1-yl]ethyl}quinazoline (CF_{cor}-325), rescued ΔF508 CFTR. Incubation of BHK cells stably expressing human ΔF508 CFTR with 1−10 μM CF_{cor}-325 resulted in maturation and delivery of a functional molecule to the cell surface as determined by the iodide efflux assay. The misprocessed CFTR mutants R258G, S945L, and H949Y were also rescued by CF_{cor}-325 in either BHK or HEK 293 cells. CF_{cor}-325 appeared to be specific for ΔF508 CFTR because another quinazoline derivative, prazosin, did not rescue the misprocessed CFTR mutants. CF_{cor}-325 could also rescue misprocessed mutants of P-gp. The compound was a P-gp inhibitor as it inhibited vinblastine-stimulated ATPase activity. P-gp-mediated vinblastine resistance was also reduced about 10-fold with 300 nM CF_{cor}-325. These results show that CF_{cor}-325 is a particularly important lead compound for treatment of CF because low concentrations can be used to rescue many misprocessed CFTR mutants.

Keywords: CFTR; cystic fibrosis; CF corrector; chemical/pharmacological chaperone; protein trafficking; drug rescue; protein folding

Introduction

Cystic fibrosis (CF) is a lethal inherited disorder that is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR).[1] The most serious clinical manifestation of CF is accumulation of thick, dehydrated mucus in the airways that ultimately results in lung failure due to chronic bacterial infections and inflammation.[2,3]

CFTR is a cAMP-dependent chloride channel located at the cell surface that regulates salt and fluid transport across the cell membrane of epithelial cells.[4] Deletion of Phe508 (ΔF508) is the most common CF mutation as it is found on at least one chromosome in 90% of affected individuals.[1] The misfolded ΔF508 CFTR protein does not reach the cell surface but is retained in an inactive form[5] in the endoplasmic reticulum (ER) by the cell's quality control mechanism and is rapidly degraded.[6−8] An important observation, however,

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is that the ΔF508 CFTR can still exhibit substantial channel activity if it can be coaxed to the cell surface by expression at low temperature (27 °C)[9] or by nonspecific osmolytes such as glycerol and TMAO.[10,11] Such methods to induce proper folding of ΔF508 CFTR are useful tools for studying CFTR in vitro but are not clinically applicable because of the conditions of hypothermia or high concentrations of osmolytes that would be required. An alternative approach to correct processing defects is to use a drug-rescue method that has been used with great success to correct processing defects in CFTR's sister protein, P-glycoprotein (P-gp).[12] Therefore, an important goal in CF research is to identify compounds that can be used at low concentrations to promote proper folding and trafficking of ΔF508 CFTR to the cell surface at physiological temperatures.

In this study we studied the ability of a novel quinazoline derivative, 4-cyclohexyloxy-2-{1-[4-(4-methoxy-benzenesulfonyl)piperazin-1-yl]ethyl}quinazoline (CF_{cor}-325), to promote maturation and trafficking of misprocessed CFTR and P-gp mutants to the cell surface.

Experimental Section

Expression of Mutants. Wild-type, ΔF508, H139R, G149R, R258G, S945L, and H949Y CFTR cDNAs were inserted into the pcDNA3 (Invitrogen, Oakville, ON) vector as described previously.[13,14] Wild-type and mutant G268V P-gp cDNAs were inserted into the pMT21 vector (Genetics Institute) as described previously.[15] Baby hamster kidney (BHK) cells stably expressing CFTR or P-gp were generated by cotransfection with cDNA and pWL-neo (Stratagene, Cedar Creek, TX). G418-resistant colonies were selected and clones expressing CFTR or P-gp were identified by subjecting the cells to immunoblot analysis with rabbit polyclonal antibody against CFTR or P-gp.[16,17] BHK cells expressing CFTR or P-gp were then expanded and used in the drug-rescue assays.

Effect of CF_{cor}-325 on Misprocessed Mutants. BHK cells stably expressing wild-type CFTR, ΔF508 CFTR, wild-type P-gp, or G268V P-gp were grown to about 50% confluence in 6-well plates in Dulbecco's modification of Eagle's medium (DMEM) with 10% (v/v) calf serum at 37 °C. For transient expression, HEK 293 cells were transfected with cDNAs of wild-type or mutant CFTR and P-gp as described previously.[18] After 24 h, medium was replaced with Opti-MEM medium (Invitrogen, Oakville, ON) containing 2% (v/v) calf serum and various concentrations of CF_{cor}-325 (Vertex Pharmaceuticals, San Diego, CA) or prazosin (Sigma-Aldrich, Mississauga, ON) at 37 °C under 5% CO_2. The cells were harvested after 24−48 h and solubilized with 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 4% (v/v) 2-mercaptoethanol) containing 50 mM EDTA. Samples were then subjected to SDS−PAGE (5.5% acrylamide gels) and immunoblot analysis with rabbit polyclonal antibody against CFTR or P-gp.[16,17]

Measurement of cAMP-Stimulated Iodide Efflux. Equivalent numbers of BHK cells stably expressing wild-type or ΔF508 CFTR were grown in triplicate to 50%

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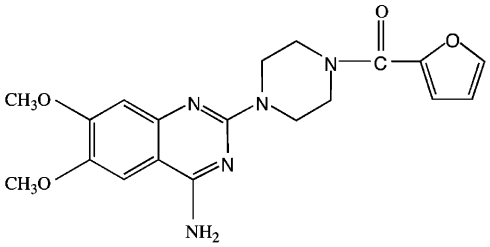
confluence in 6-well plates. The medium was then replaced with Opti-MEM with 2% (v/v) calf serum and with or without 3 μM CF_{cor}-325. After 48 h at 37 °C, the medium was removed and the cells were loaded with sodium iodide for 1 h at 20 °C. Iodide efflux was then monitored using an iodide-specific electrode (Analytical Sensors and Instruments, Sugar Land, TX) after stimulation with 10 μM forskolin in the presence of 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.5 mM chlorophenylthio-cAMP as described previously.[16]

Measurement of P-gp-Mediated Drug Resistance. BHK cells stably expressing wild-type P-gp were incubated in Opti-MEM with 2% (v/v) calf serum and various concentrations (0−1 μM) of vinblastine in the absence or presence of 300 nM CF_{cor}-325. After 6 days at 37 °C, cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously.[19]

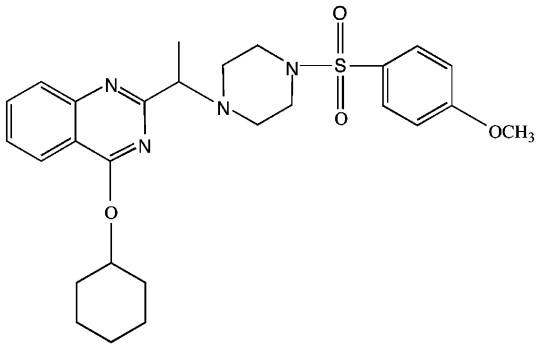
Isolation of P-gp Mutants and Measurement of Drug-Stimulated ATPase Activity. Histidine-tagged P-gp was isolated by nickel-chelate chromatography as described previously.[20,21] An aliquot of the isolated histidine-tagged P-gp was then mixed with an equal volume of 10 mg/mL sheep brain phosphatidylethanolamine (Type II-S, Sigma) that had been washed and suspended in TBS. The P-gp/lipid mixture was then sonicated, and a sample of the mixture was incubated with various concentrations (0−1 mM) of CF_{cor}-325 for 15 min at 20 °C. ATPase activity was initiated by addition of an equal volume of ATPase buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM ATP with and without 100 μM vinblastine. A final concentration of 50 μM vinblastine was saturating and resulted in maximal activation.[22] The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate released was determined.[23]

Results

The use of specific chemical/pharmacological chaperones to correct folding and trafficking defects (protein kinesis) was first demonstrated with misprocessed mutants of P-gp,



Prazosin:
1-(4-amino-6,7-dimethoxy-2-quinozolinyl)-4-(2-furoyl)piperazine



CFcor-325:
4-cyclohexyloxy-2-{1-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-ethyl}-quinazoline

Figure 1. Structures of the quinazoline derivatives prazosin and CF_{cor}-325.

a sister protein of CFTR.[12] Deletion of Tyr490 (ΔY490) in P-gp (equivalent to Phe508 in CFTR) also caused the mutant protein to be retained in the ER.[24] The folding defect in ΔY490 P-gp, however, was corrected by carrying out expression in the presence of drug substrates and modulators of P-gp. The rescued ΔY490 P-gp was trafficked to the cell surface and was functional. Unfortunately, compounds such as capsaicin, cyclosporin A, verapamil, or vinblastine that are specific for P-gp could not rescue ΔF508 CFTR.[12,25]

High-throughput screening has been used to identify potential candidate compounds that may rescue ΔF508 CFTR.[26] Recently, Vertex Pharmaceuticals (San Diego, CA) with support from the Cystic Fibrosis Foundation used high-throughput assays to identify compounds that could be useful in CFTR research.[27] CF_{cor}-325, a quinazoline derivative that is similar to prazosin (Figure 1), was identified as a potential

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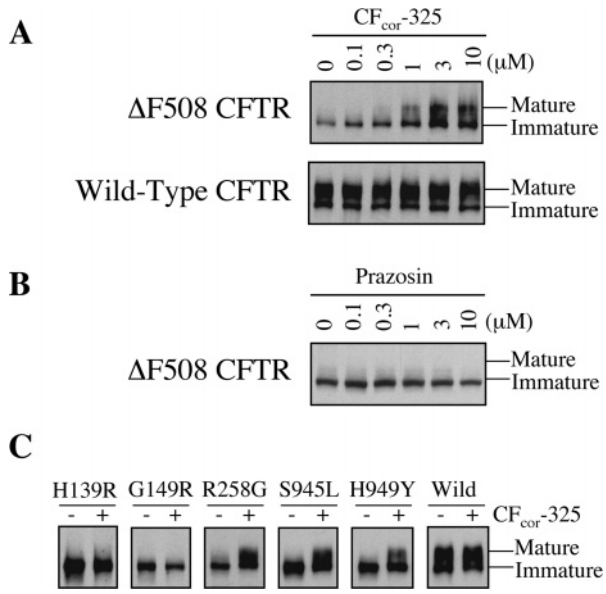

Figure 2. Effect of quinazoline derivatives on maturation of misprocessed CFTR mutants. BHK cells stably expressing wild-type or ΔF508 CFTR were incubated for 48 h in the presence of 0−10 μM CF_{cor}-325 (A) or prazosin (B). (C) BHK cells expressing misprocessed CFTR mutants H139R, G149R, R258G, S945L, H949Y, or wild-type CFTR were incubated for 48 h with (+) or without (−) 3 μM CF_{cor}-325. Whole cell extracts were subjected to immunoblot analysis with a rabbit polyclonal antibody against CFTR. The positions of the mature and immature CFTRs are indicated.

corrector of the defects in ΔF508 CFTR (unpublished structure, Vertex Pharmaceuticals, San Diego, CA).

The effect of CF_{cor}-325 on maturation of human wild-type and ΔF508 CFTRs stably expressed in BHK cells was tested. BHK cells were used because they do not express detectable endogenous CFTR. CFTR uses two N-glycosylation sites, and its maturation can be monitored by SDS−PAGE as the core-glycosylated immature CFTR has a higher mobility than the mature form of the protein. Accordingly, BHK cells stably expressing ΔF508 CFTR were incubated with various concentrations of CF_{cor}-325 for 48 h. Whole cell SDS extracts were then subjected to immunoblot analysis. Figure 2A shows that CF_{cor}-325 promoted maturation of ΔF508 CFTR in a dose-dependent manner. In the absence of CF_{cor}-325, the immature ΔF508 CFTR migrates with an apparent mass of 160 kDa. The presence of mature ΔF508 CFTR with an apparent mass of 190 kDa could be detected at 1 μM CF_{cor}-325, with maximum levels of the mature protein observed at 3 and 10 μM CF_{cor}-325. By contrast, CF_{cor}-325 had little effect on BHK cells stably expressing wild-type CFTR (Figure 2A). Concentrations of CF_{cor}-325 greater than 10 μM inhibited cell growth (data not shown). These results show that rescue of ΔF508 CFTR by CF_{cor}-325 occurred by an increase in folding efficiency rather than through an increase in transcription of the CFTR gene as seen with sodium butyrate.[28]

To test whether drug rescue of ΔF508 CFTR by CF_{cor}-325 was specific we used prazosin as a control. Prazosin,

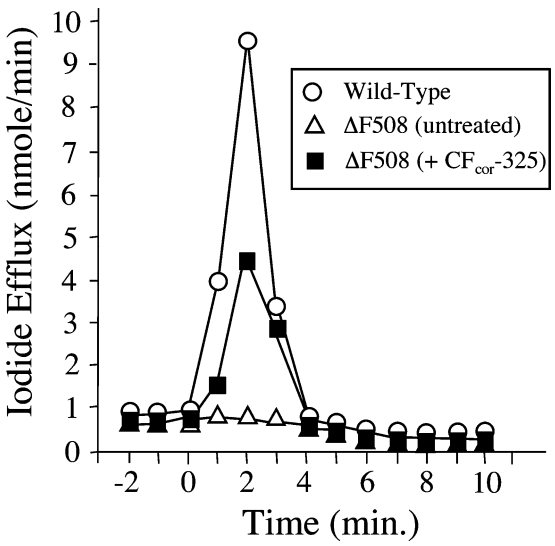

Figure 3. Measurement of cAMP-stimulated iodide efflux. Iodide efflux assays were performed in BHK cells stably expressing wild-type or ΔF508 CFTR and treated with or without 3 μM CF_{cor}-325 for 48 h as described in the Experimental Section. Time 0 is the start of stimulation through addition of 10 μM forskolin.

an antihypertensive agent, is also a quinazoline derivative (Figure 1). It is relatively hydrophobic and also inhibits cell growth at concentration >10 μM (data not shown). BHK cells stably expressing ΔF508 CFTR were grown for 48 h in the presence of 0−10 μM prazosin. Figure 2B shows that prazosin did not promote maturation of the protein.

We then tested whether the rescued ΔF508 CFTR was trafficked to the cell surface in an active form where channel activity could be measured. Iodide efflux assays were used rather than chloride channel measurement because very few channels other than CFTR can conduct iodide ions.[29] Accordingly, we measured cAMP-stimulated iodide efflux by CFTR. BHK cells stably expressing ΔF508 CFTR were treated with or without 3 μM CF_{cor}-325 for 48 h. The cells were then loaded with sodium iodide, and iodide efflux was measured. BHK cells stably expressing ΔF508 CFTR grown without CF_{cor}-325 showed no stimulation of iodide efflux in the presence of forskolin (Figure 3). The addition of forskolin

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to BHK cells expressing wild-type CFTR, however, resulted in a large stimulation of iodide efflux that reached a maximum after 2 min. Similarly, BHK cells stably expressing ΔF508 CFTR that were treated with CF_{cor}-325 also exhibited maximum iodide activity at 2 min, but maximum efflux activity was about 30% of that observed with wild-type CFTR (Figure 3). These results show that CF_{cor}-325 increased the number of active ΔF508 CFTR channels at the cell surface.

Iodide efflux activity was not significantly inhibited or stimulated when 3 μM CF_{cor}-325 was included in the sodium iodide loading and wash buffers (data not shown). It is possible, however, that CF_{cor}-325 could have subtle effects on the gating properties of CFTR that could not be detected in the iodide efflux assay.

The ΔF508 mutation is in the NH_2-terminal nucleotide-binding domain (NBD). We wanted to test whether CF_{cor}-325 could promote maturation of other misprocessed CFTR mutants with mutations in other domains. BHK cells expressing mutants H139R, G149R, and R258G in the first transmembrane domain (TMD1)[30] or mutants S945L and H949Y in TMD2[13] were treated with or without 3 μM CF_{cor}-325 for 48 h. Whole cell SDS extracts were then subjected to immunoblot analysis. The presence of CF_{cor}-325 significantly enhanced maturation of mutants R258G, S945L, and H949Y (Figure 2C). By contrast, CF_{cor}-325 had little effect on mutants H139R or G149R. Similar results were observed when HEK 293 cells were transiently transfected with these mutant cDNAs and treated with CF_{cor}-325 (data not shown). Therefore, CF_{cor}-325 can promote maturation of some CFTR mutants that have mutations in different domains in different cell lines.

An explanation for the ability of CF_{cor}-325 to promote folding and trafficking of misprocessed CFTR mutants is that it diffused into the ER and directly interacts with the mutant protein during biogenesis as observed with P-gp.[31,32] Another explanation is that CF_{cor}-325 has a nonspecific effect on protein folding similar to that observed when low temperature or osmolytes such as glycerol or trimethylamine *N*-oxide are used to rescue misfolded proteins.[9,10] To test whether CF_{cor}-325 had a direct or indirect effect on protein folding, we used the P-gp processing mutant G268V as a control.[33] We used the G268V P-gp mutant, rather than mutant ΔY490 P-gp (equivalent to ΔF508 in CFTR), as a control because maturation of the G268V mutant is not promoted by growth at low temperature (27 °C) or by osmolytes such as glycerol and trimethylamine *N*-oxide (unpublished observations). Maturation of mutant G268V to yield active enzyme at the cell surface, however, can be achieved by carrying out expression in the presence of drug substrates or modulators.[34] Accordingly, BHK cells stably expressing P-gp mutant G268V were incubated with 0−10 μM CF_{cor}-325 for 48 h. Immunoblot analysis of whole cell SDS extracts showed that the presence of 3−10 μM CF_{cor}-325 caused a large increase in the relative amount of the mature 170 kDa protein (Figure 4A). These results show that the concentration of CF_{cor}-325 in the ER was sufficient to act directly on the protein.

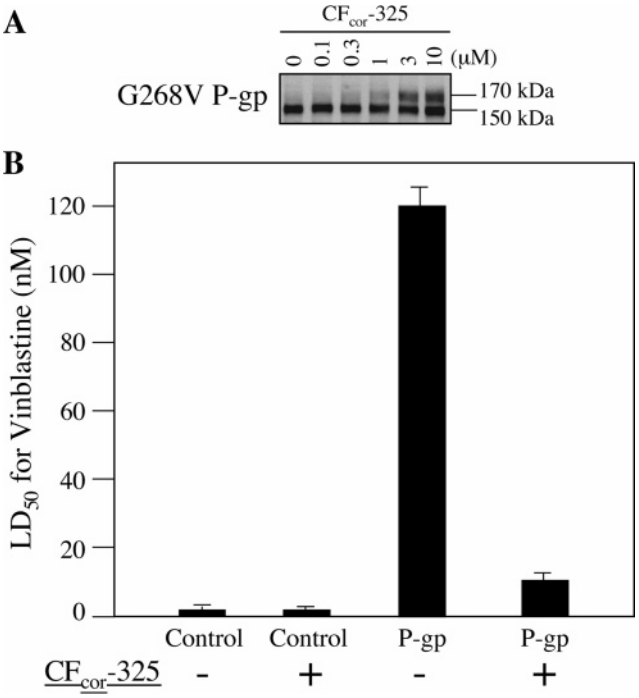

Figure 4. Effect of CF_{cor}-325 on expression of misprocessed P-gp mutant G268V and on P-gp-mediated drug resistance. (A) BHK cells stably expressing P-gp mutant G268V were treated for 48 h with 0−10 μM CF_{cor}-325. Whole cell extracts were then subjected to immunoblot analysis with rabbit polyclonal antibody against P-gp. The positions of the mature (170 kDa) and immature (150 kDa) P-gps are shown. (B) BHK cells stably expressing wild-type P-gp (P-gp) or mock-transfected BHK cells (control) were incubated with 0−1 μM vinblastine in the presence (+) or absence (−) of 300 nM CF_{cor}-325. After 6 days, the number of viable cells was determined. The concentration of vinblastine that inhibited cell growth by 50% (LD_{50}) was then determined. Each value is the average of triplicate assays.

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The ability of CF_{cor}-325 to rescue P-gp mutant G268V suggested that it was a substrate or modulator of P-gp. We then tested the ability of CF_{cor}-325 to stimulate the ATPase activity of P-gp. Most drug substrates of P-gp will stimulate or inhibit the ATPase activity of P-gp.[35] Histidine-tagged wild-type P-gp was isolated, mixed with lipid, and then incubated with various concentrations of CF_{cor}-325. The ATPase activity was then measured in the presence or absence of vinblastine. The ATPase activity of P-gp was not stimulated with 1 mM CF_{cor}-325, but the vinblastine-stimulated ATPase activity was inhibited by CF_{cor}-325 (IC_{50} of 7.4 μM) (data not shown). The inhibitory nature of CF_{cor}-325 was further confirmed by its ability to inhibit P-gp-mediated drug resistance. BHK cells stably expressing wild-type P-gp were incubated with various concentrations (0−1 μM) of vinblastine and with or without 300 nM CF_{cor}-325. Figure 4B shows that the presence of CF_{cor}-325 caused about a 10-fold reduction in the ability of P-gp expressing cells to confer resistance to vinblastine.

Discussion

The results show that the presence of relatively low concentrations (1−10 μM) of CF_{cor}-325 could enhance maturation of both misprocessed mutants of CFTR and P-gp. It was possible to promote maturation of some mutants that had mutations in different domains of CFTR including NBD1 (ΔF508), TMD1 (R258G in the second intracellular loop), and TMD2 (S945L and H949Y in the third intracellular loop). All of these mutants have been shown to possess channel activity when they reach the cell surface, although they often show altered gating properties.[9,13,30]

The iodide efflux activity of mature ΔF508 CFTR rescued with CF_{cor}-325 did not reach the levels observed with wild-type CFTR (Figure 3) because maturation was still less efficient than that of wild-type CFTR. Could partial rescue of misprocessed CFTR mutants be useful in the treatment of CF? Evidence from several studies suggests that even a partial correction of the processing defects would be useful. It has been reported that low levels (10%) of normal CFTR RNA were sufficient for normal lung function[36] and that patients expressing only about 4% of the normal CFTR mRNA showed a very mild CF phenotype.[37] Studies on cultured cells showed that only 6−10% of the cells need to express CFTR in order to correct the electrophysiological parameters in CF epithelia.[38]

Other compounds have been reported to promote maturation of ΔF508 CFTR. These include the substituted benzo[*c*]-quinolizinium compounds,[39] thapsigargin,[40] and curcumin.[41] Drug rescue of ΔF508 CFTR with benzo[*c*]quinolizinium compounds, however, requires concentrations that are about 100-fold higher (250−500 μM) than that needed for CF_{cor}-325, while rescue with thapsigargin and curcumin could not be repeated by other investigators.[25,42,43] A possible explanation for the observation that curcumin treatment increased cAMP-stimulated iodide efflux in BHK cells expressing ΔF508 CFTR[41] was that the compound can directly stimulate CFTR channels that were already present at the cell surface.[44]

We were unable to induce maturation of misprocessed CFTR mutants that had the mutations H139R or G149R in the first intracellular loop. It is possible that introduction of a bulky and highly charged residue at either position causes a large thermodynamic hurdle in the folding process.[45] In our models for the effect of processing mutations on the folding of P-gp[46] or CFTR,[16] we proposed that processing mutations transiently trap the proteins in a prefolded state resulting in incomplete domain−domain interactions. An important interaction in P-gp appears to be the interaction between NBD1 and the first cytoplasmic loop of TMD1.[46] Recent studies on ΔF508 CFTR suggest that the presence of localized misfolding in CFTR also affects domain−domain interactions.[47−49] Perhaps introduction of arginines

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in the first cytoplasmic loop (H139R or G149R) prevents the establishment of these interactions.

In summary, this is the first report showing that CF_{cor}-325 is a potent chemical chaperone for ΔF508 and other processing CFTR mutants. It is also a potent inhibitor of P-gp. Inhibition of P-gp may be beneficial since P-gp normally decreases the bioavailability of drugs. An interesting observation was that 300 nM CF_{cor}-325 was sufficient to completely inhibit P-gp ATPase activity, whereas CFTR iodide efflux activity was observed in the presence of 3 μM CF_{cor}-325. Therefore CF_{cor}-325 does not block the CFTR channel. This makes it a very useful lead compound for the development of a drug-rescue approach for treating CF. Numerous different derivatives of CF_{cor}-325 could be synthesized that may be more potent for rescuing ΔF508 CFTR such that even lower concentrations of drugs are required for rescuing the misprocessed CFTRs.

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Abbreviations Used

BHK, baby hamster kidney cells; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CF_{cor}-325, 4-cyclohexyloxy-2-{1-[4-(4−methoxy-benzenesulfonyl)piperazin-1-yl]ethyl}quinazoline; ER, endoplasmic reticulum; P-gp, P-glycoprotein; HEK, human embryonic kidney; NBD, nucleotide-binding domain; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMD, transmembrane domain.

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