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Rescue of Folding Defects in ABC Transporters Using Pharmacological Chaperones

Tip W. Loo,[1,2] M. Claire Bartlett,[1,2] and David M. Clarke[1,2,3]

The ATP-binding cassette (ABC) family of membrane transport proteins is the largest class of transporters in humans (48 members). The majority of ABC transporters function at the cell surface. Therefore, defective folding and trafficking of the protein to the cell surface can lead to serious health problems. The classic example is cystic fibrosis (CF). In most CF patients, there is a deletion of Phe508 in the CFTR protein (ΔF508 CFTR) that results in defective folding and intracellular retention of the protein (processing mutant). A potential treatment for most patients with CF would be to use a ligand(s) of CFTR that acts a pharmacological chaperone to correct the folding defect. The feasibility of such an approach was first demonstrated with the multidrug transporter P-glycoprotein (P-gp), an ABC transporter, and a sister protein of CFTR. It was found that P-gps with mutations at sites equivalent to those found in CFTR processing mutants were rescued when they were expressed in the presence of drug substrates or modulators of P-gp. These compounds acted as pharmacological chaperones and functioned by promoting interactions among the various domains in the protein during the folding process. Several groups have attempted to identify compounds that could rescue the folding defect in ΔF508 CFTR. The best compound identified through high-throughout screening is a quinazoline derivative (CFcor-325). Expression of ΔF508 CFTR as well as other CFTR processing mutants in the presence of 1 μM CFcor-325 promoted folding and trafficking of the mutant proteins to the cell surface in an active conformation. Therefore, CFcor-325 and other quinazoline derivates could be important therapeutic compounds for the treatment of CF.

KEY WORDS: Cystic fibrosis; CFTR; pharmacological chaperones; drug rescue; P-glycoprotein; protein misfolding.

CYSTIC FIBROSIS AND CFTR PROCESSING MUTATIONS

Cystic fibrosis (CF) is a lethal inherited disorder. It is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al*., 1989). It is a relatively common genetic defect in Caucasians as 1 in 25 persons is a carrier and the disease affects 1 in 2500 live births (Boat and Cheng, 1989). The most serious clinical manifestation in CF is accumulation of thick dehydrated mucus in the airways. Death in CF patients is often from lung failure due to chronic bacterial infections and inflammation (Goodman and Percy, 2005; Guggino and Banks-Schlegel, 2004).

In normal epithelial cells that line the airways, CFTR is at the cell surface and helps to modulate hydration of the mucus layer. It is a cAMP-dependent chloride channel that helps to regulate salt and fluid transport across the plasma membrane. Deletion of residue Phe508 (ΔF508) is the most common mutation in CF patients, as it is found on at least one chromosome in 90% of affected individuals. The ΔF508 mutation causes misfolding of CFTR such

[1] Department of Medicine, University of Toronto, Rm. 7342, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8.
[2] Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8.
[3] To whom correspondence should be addressed; e-mail: david.clarke@utoronto.ca.

that the protein is retained in the endoplasmic reticulum (ER) and rapidly degraded (Cheng *et al*., 1990; Kartner *et al*., 1992).

An important observation with respect to developing a treatment for CF was that the ΔF508 CFTR mutant still exhibited substantial chloride channel activity if it was coaxed to the cell surface by expression at low temperature (Denning *et al*., 1992) or expression in the presence of osmolytes such as glycerol or TMAO (Brown *et al*., 1996; Sato *et al*., 1996). Unfortunately, it is not practical to cool CF patients to 27°C or to infuse them with high concentrations of osmolytes (molar amounts) required to rescue ΔF508 CFTR. Studies on CFTR's sister protein, P-glycoprotein (P-gp), however, suggest that it may be possible to identify ligands (substrates/modulators/inhibitors) that can be used at relatively low concentrations (less than 10 μM) as chemical/pharmacological chaperones to correct the folding defects in the protein (Loo and Clarke, 1997a).

CORRECTING FOLDING DEFECTS IN P-gp PROCESSING MUTANTS USING A DRUG-RESCUE APPROACH

A useful model system for studying folding and maturation of CFTR and other ABC transporters is the multidrug resistance P-gp (ABCB1). P-gp is an ATP-dependent drug pump located in the plasma membrane of epithelial cells of organs such as the intestine, liver, kidney, and blood–brain/testes barriers. It transports a variety of structurally diverse compounds out of the cell. Its physiological role is unknown, but it may protect the organism from toxins in the diet and environment (Loo and Clarke, 1999c). Unfortunately, many therapeutic drugs used in cancer and HIV/AIDS are also substrates of P-gp (Lee *et al*., 1998).

P-gp and CFTR are members of the ATP-binding cassette (ABC) family of transporters. Cloning and sequencing of P-gp and CFTR proteins indicated that both protein are structurally similar (Chen *et al*., 1986; Riordan *et al*., 1989). Both proteins have two halves that are joined together by a linker region. Each half has a transmembrane domain (TMD) containing six predicted transmembrane (TM) segments followed by a nucleotide-binding domain (NBD) (Fig. 1A). CFTR has an addition regulatory (R) domain immediately following the NBD1, although the protein can still function when the R domain is deleted (Rich *et al*., 1991).

Mutations in P-gp have been identified in the TM segments, loops connecting the TM segments, in the linker region and within the NBDs that affect processing of P-

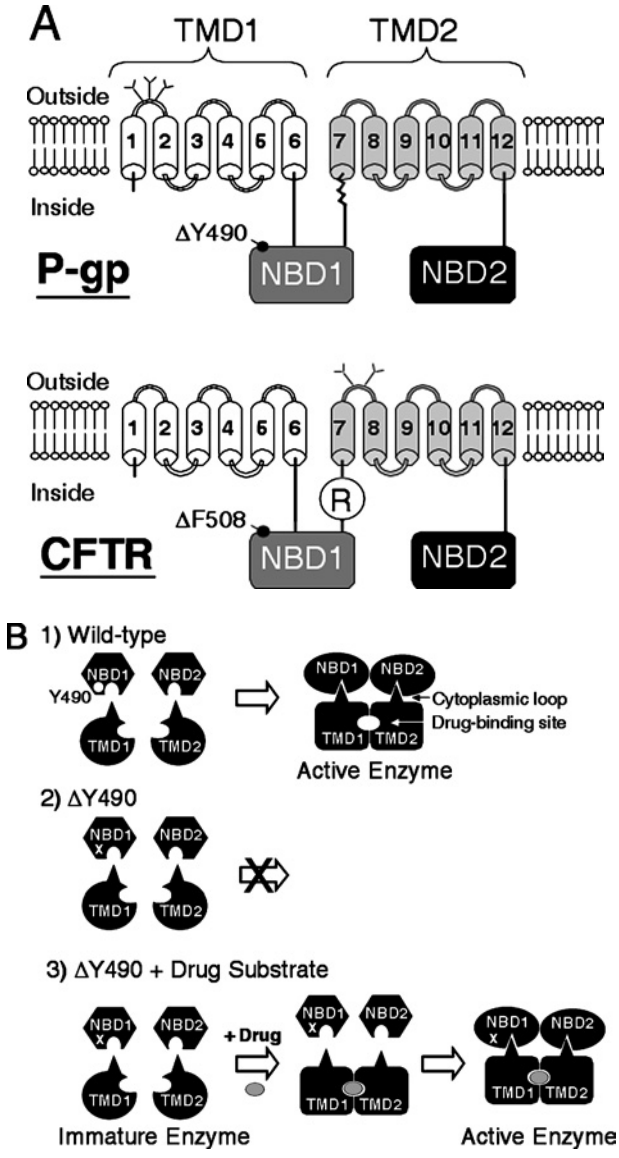

Fig. 1. Models of P-gp, CFTR, and P-gp maturation. (A) The 12 TMs of P-gp and CFTR are shown as numbered cylinders. The branched lines represent glycosylation sites while the zigzag lines represent the linker region. TMD, NBD, and R represent the transmembrane, nucleotide-binding, and regulatory domains, respectively. The positions of F508 in CFTR and the equivalent residue in P-gp (Y490) are indicated. (B) Panel 1 shows that wild-type P-gp undergoes efficient maturation because all four domains interact properly during protein folding. In panel 2, the loss of Y490 in NBD1 (X) inhibits protein folding by reducing the efficiency of NBD1–TMD1 interactions and subsequent TMD1–TMD2 interactions. The drug substrate (panel 3) acts as a scaffold to stabilize the "native" structure that exists transiently in the mutant ΔY490 protein and results in proper interactions between TMD1–TMD2, TMD–NBD, and NBD–NBD. The result is an active enzyme.

gp (Loo and Clarke, 1997a). These mutants are retained in the ER as core-glycosylated proteins and are rapidly degraded (Loo and Clarke, 1994). When the processing mutants were expressed in the presence of drug substrates or modulators such as verapamil, cyclosporin A, vinblastine, capsaicin, or nonylphenol ethoxylates; however, the mutant proteins exited the ER and their carbohydrate moieties were modified in the Golgi before delivery to the cell surface as functional transporters (Loo and Clarke, 1997a, 1998a). Mutant ΔY490 P-gp was particularly interesting because residue Y490 is at the equivalent position to ΔF508 in CFTR (Hoof *et al*., 1994). The ΔY490 P-gp mutant was also retained in the ER. It was however, correctly targeted to the cell surface in an active conformation after expression in the presence of a drug substrate (Loo and Clarke, 1997a). Therefore, understanding how the ΔY490 mutation affects P-gp maturation and how drug substrates (chemical/pharmacological chaperones) can rescue the folding defects may provide important clues in the development of a therapy for treating CF.

The first clue about how processing mutations affect P-gp was the observation that the immature core-glycosylated protein was relatively more sensitive to protease digestion than the mature full-glycosylated P-gp (Loo and Clarke, 1998c). The immature mutant protein was about 100-fold more sensitive to trypsin compared to the mature form of wild-type P-gp. The increased sensitivity to protease digestion was not due to the mutation per se because the immature form of wild-type P-gp was also about 100-fold more sensitive to trypsin. The mature enzyme, however, was folded in a more compact protease-resistant conformation during maturation and delivery to the cell surface. The misprocessed mutant P-gp could also be converted to the protease-resistant conformation by carrying out expression in the presence of drug substrate. The implications of these observations were that the processing mutant was structurally similar to the immature wild-type P-gp in the ER and that both immature forms were present in a loosely folded protease sensitive conformation.

Another difference observed between the mature and immature forms of P-gp was that the immature wild-type and mutant P-gps were inactive (Loo and Clarke, 1999a). It was found that the immature core-glycosylated P-gp showed no detectable activity when it was prevented from reaching the cell surface by introduction of a processing mutation or by carrying out expression in the presence of the proteasome inhibitor, MG-132. Expression of P-gp (Loo and Clarke, 1998b) or CFTR (Jensen *et al*., 1995) in the presence of MG-132 inhibits maturation of both proteins.

Maturation of CFTR shows many similarities to P-gp. The presence of MG-132 during expression of CFTR inhibits maturation of the protein and results in an inactive CFTR that is more sensitive to trypsin than the wild-type CFTR (Chen *et al*., 2000). Similarly, Zhang *et al*. (1998) compared the protease sensitivities of wild-type and ΔF508 CFTRs and showed that the ΔF508 CFTR proteolytic cleavage patterns were indistinguishable from those of the early folding intermediates of wild-type CFTR. Therefore, the ΔF508 mutation in CFTR also appears to trap CFTR as an early folding intermediate.

DETERMINING THE MINIMUM SIZE OF P-gp THAT CAN BE RESCUED BY DRUG SUBSTRATE

We were interested in determining the minimum structure in P-gp that could be rescued by drug substrates or modulators. Since P-gp has two homologous halves with the NH_2-terminal half showing 43% amino acid identity with the COOH-terminal half, we tested whether half-molecules expressed as separate polypeptides could be rescued by drug substrates. Maturation of the NH_2- or COOH− half-molecules was not observed when either half-molecule was expressed in the absence or presence of drug substrate (Loo and Clarke, 1998c). By contrast, maturation of the half-molecules of wild-type P-gp was detected when they were contemporaneously expressed in the same cell in the presence of drug substrate. These results indicated that interaction of both halves of P-gp was required during synthesis for proper folding of the molecule. Interaction between the two halves of P-gp was further demonstrated by placing a histidine-tag at the COOH-terminal end of one half molecule and co-expressing it with the second half-molecule containing no histidine tag. After nickel-chelate chromatography, the wild-type half-molecule containing no histidine tag was also recovered together with the histidine-tagged half-molecule (Loo and Clarke, 1998c). The presence of a processing mutation in either half-molecule (N-half (G268V) or COOH-half (A841L)) resulted in the loss of interactions between the half-molecules. Interaction between the two halves in these mutants was restored, however, if both halves were co-expressed in the presence of drug substrate. These studies show that processing mutations disrupt interactions between the two halves of P-gp while expression in the presence of drug substrate restores these interactions.

Extensive cysteine-scanning mutagenesis studies on P-gp and reaction of the mutants with thiol-reactive substrate analogues indicate that the drug-binding sites are located at the interface between the TMDs (Loo

et al., 2003a,b,c; Loo and Clarke, 1997b, 1999b, 2000a, 2001a,b, 2002a,b). Therefore, it was predicted that drug substrates promote interactions between TMD1 and TMD2. Indeed, the NBDs of P-gp are not essential because a deletion mutant of P-gp lacking both NBDs still bound drug-substrates. This TMD1+TMD2 deletion mutant is retained in the ER, but is trafficked to the cell surface in a protease-resistant conformation when expressed in the presence of a drug substrate (Loo and Clarke, 1999d). Both TMDs, however, are required for it to be rescued with drug substrate. There was no evidence of drug-rescue when either TMD1 or TMD2 was expressed separately (quarter molecules) (Loo and Clarke, 1998c). Expression of both TMD1 and TMD2 quarter molecules together in the same cell in the presence of drug substrate, however, promoted interaction between the two molecules resulting in a protease-resistant conformation.

EFFECT OF PROCESSING MUTATIONS AND DRUG SUBSTRATES ON PACKING OF TM SEGMENTS

An explanation for the ability of drug substrates to rescue processing P-gp mutants is that they promote packing of the TM segments. Some contact points between the two TMDs have been mapped using cysteine mutagenesis and disulfide cross-linking analysis (Loo and Clarke, 2000b). For example, cysteines introduced into TMs 4 or 5 (TMD1) were cross-linked to cysteines in TM12 (TMD2) with a zero-length cross-linker (copper phenanthroline). Cross-linking between TMD1 and TMD2 was inhibited however, when the ΔY490 mutation (equivalent position to ΔF508 in CFTR) was introduced into the TM4/TM12 or TM5/TM12 double cysteine mutants. Cross-linking of the ΔY490 double cysteine mutant was restored when it was expressed in the presence of drug substrate (Loo *et al*., 2002a).

Why does a processing mutation in NBD (ΔY490) alter interactions between TMD1 and TMD2 in P-gp? A possible explanation was that the ΔY490 mutation may lie at the interface between NBD1 and TMD1, and mutations that affect this interaction could influence packing of the TM segments. It has been shown that NBD1 and TMD1 can interact when expressed as separate polypeptides (Loo and Clarke, 1995). Therefore, cross-linking was attempted between a cysteine introduced at position 490 and another cysteine at various positions in the first cytoplasmic loop of TMD1. It was found that Cys 490 (NBD1) could be cross-linked with Cys184 (first cytoplasmic loop) with copper phenanthroline (Loo *et al*., 2002a). The crystal structures of the bacterial ABC transporters such as BtuCD show similar interactions between NBD1 and the first cytoplasmic loop (Locher *et al*., 2002). Therefore, deletion of residue Y490 may disrupt folding of the molecule because it interferes with the association of NBD1 and TMD1.

Another domain–domain interaction that can be disrupted by the presence of processing mutations is the interaction between NBD1 and NBD2. Disulfide cross-linking analysis studies have shown that a cysteine introduced into the Walker A (GNSGCGKS in NBD1; GSSGCGKS in NBD2) site of one NBD can be efficiently cross-linked to a cysteine introduced into the LSGGQ signature sequence of the other NBD with copper phenanthroline (Loo *et al*., 2002b). The presence of a processing mutation (in TMD1, NBD1, linker region, or TMD2) inhibited cross-linking between the NBDs (Loo *et al*., 2004a). Cross-linking between the NBDs was restored however, when the processing mutants were first rescued by carrying out expression in the presence of drug substrate.

These results on P-gp processing mutants suggest that processing mutations disrupt domain–domain (TMD–TMD, TMD–NBD, and NBD–NBD) interactions resulting in a mutant protein that is trapped in the ER as a loosely folded intermediate. We hypothesize that the common drug-binding pocket and proper domain–domain interactions must exist transiently during the folding of the misprocessed P-gps. Drug substrates must rescue these transient conformations by acting as a scaffold to stabilize these near-native conformations during folding and allow proper domain–domain interactions to occur. The near-native conformations likely exist transiently because misprocessed histidine-tagged P-gp mutants (grown in the absence of drug substrate) that are isolated by nickel-chromatography cannot subsequently be rescued by addition of drug substrate (unpublished data).

A model for the effect of processing mutations and rescue by drug substrate is shown in Fig. 1B. P-gp is first synthesized in the ER where each domain can fold independently. This is supported by the finding that each domain of P-gp can be stably expressed and in a conformation that can still bind ligand (Baubichon-Cortay *et al*., 1994; Dayan *et al*., 1996; Loo and Clarke, 1995). In many bacterial ABC transporters, the four domains are expressed as separate polypeptides that then associate to form an active transporter (Altenberg, 2003). In our model, the four domains form a transiently loosely folded structure that associate with chaperones such as calnexin and Hsp 70 (Loo and Clarke, 1994, 1995). In wild-type P-gp, the protein then undergoes superfolding to form native domain–domain contacts to yield a functional molecule

that is then trafficked to the cell surface. The presence of a processing mutation, however, causes the protein to become stuck in the ER in the loosely folded conformation that is eventually degraded (Loo and Clarke, 1998b). In the presence of drug substrate however, the substrate enters the drug-binding pocket and induces packing of the TM segments in TMD1 and TMD2. Correct packing of the TM segments will then promote NBD–TMD interactions through contacts with the intracellular loops that in turn promote NBD1–NBD2 interactions. The stabilized protein will be recognized as being correctly folded by the cell's quality control mechanism and the molecule is trafficked to the cell surface.

Recent work on CFTR is consistent with the above model for P-gp folding (Fig. 1B). Disulfide cross-linking analysis studies have shown that the ΔF508 mutation altered cross-linking between cysteines placed in TMD1 and TMD2 of CFTR (Chen *et al*., 2004). Recent crystal structure studies on NBD1 of CFTR showed that the ΔF508 mutation causes only minor structural changes in the molecule. The helix containing the ΔF508 mutation was located on the surface of the molecule and close to the predicted NBD1–TMD1 interface (Lewis *et al*., 2004, 2005).

CORRECTION OF FOLDING DEFECTS IN CFTR PROCESSING MUTANTS USING A PHARMACOLOGICAL CHAPERONE

The effects of processing mutations of the maturation characteristics of P-gp and CFTR show many similarities. Therefore, it should be possible to identify a pharmacological chaperone for rescuing CFTR processing mutants as achieved for P-gp. Unfortunately, compounds such as capsaicin, cyclosporin A, verapamil, or vinblastine that are specific for P-gp could not rescue ΔF508 CFTR (Loo and Clarke, 1997a).

A major problem in the drug-rescue of ΔF508 CFTR is identifying compounds that bind CFTR with relatively high affinities. A solution for identifying compounds that could efficiently rescue ΔF508 CFTR would be through high-throughput screening of thousands of compounds (Van Goor *et al*., 2004). One potential corrector that was identified was a quinazoline derivative termed CF_{cor}-325 (Vertex Pharmaceuticals, San Diego, CA). Expression of ΔF508 CFTR as well as other CF-associated CFTR processing mutants in the presence of relatively low concentrations (1–10 μM) of CF_{cor}-325 promoted maturation of the mutant proteins and trafficking to the cell surface in an active form (Loo *et al*., 2005). The channel activity (iodide efflux assay) was not as high as that observed with wild-type CFTR, because only 25–30% of the misprocessed ΔF508 CFTR was rescued by CF_{cor}-325. How much CFTR at the cell surface would be needed to have a major beneficial effect on CF patients? It has been reported that even 10% of normal CFTR RNA was sufficient for normal lung function (Chu *et al*., 1993) and that patients expressing only about 4% of normal CFTR mRNA showed very mild symptoms of CF (Highsmith *et al*., 1997). Studies on cultured cells showed that only 6–10% of cells need to express CFTR to correct the electrophysiological parameters in CF epithelia (Johnson *et al*., 1992).

Other compounds such as benzo(c)quinolizinium compounds (Dormer *et al*., 2001), doxorubicin (Maitra *et al*., 2001), thapsigargin (Egan *et al*., 2002), curcumin (Egan *et al*., 2004), and sildenafil (Dormer *et al*., 2005) have also been reported to promote maturation of ΔF508 CFTR. The benzo(c)quinolizinium compounds and sildenafil, however, appear to be 100-fold less potent than CF_{cor}-325 while maturation of ΔF508 CFTR by doxorubicin was reported to occur at a single dose of 0.25 μM. Rescue with thapsigargin or curcumin could not be reproduced by several investigators (Dragomir *et al*., 2004; Loo *et al*., 2004b; Song *et al*., 2004).

A surprising finding with CF_{cor}-325 was that it could also rescue processing mutants of P-gp (Loo *et al*., 2005). The compound interacted directly with P-gp because it inhibited the ability of P-gp to confer resistance to cytotoxic compounds as well as its verapamil-stimulated ATPase activity. By contrast, CF_{cor}-325 did not appear to inhibit CFTR function. The ability of CF_{cor}-325 to rescue CFTR processing mutants without inhibiting its activity makes it a valuable lead compound. Derivatives of CF_{cor}-325 may yield more potent pharmacological chaperones for ΔF508 CFTR.

There are several potential limitations to the use of pharmacological chaperones for rescuing misfolded proteins. The first is identification of ligands that bind the protein with relatively high affinity. The ligand must then be able to penetrate the cell to reach the ER in sufficient concentrations. Finally, processing mutations that affect the drug-binding site on the protein or cause total misfolding of the protein such that the drug-binding site does not exist transiently are unlikely to be rescued with chemical chaperones. Those processing mutations within the drug-binding site that affect the affinity of the protein will require significantly higher concentrations of ligands that may be toxic to the cell.

In summary, the discovery that substrates/modulators of P-gp can prevent protein misfolding has been applied to correcting folding defects in many other soluble/membrane proteins. Table I lists some of the misfolded proteins that can be rescued with ligands acting as specific chemical/pharmacological chaperones.

Table I. Some Misfolded Proteins Rescued With Specific Chemical Chaperones

Protein	Ligands	Reference
P-glycoprotein (P-gp, ABCB1)	Cyclosporin, capsaicin, vinblastine, verapamil, nonylphenol ethoxylates	Loo and Clarke (1997a, 1998a)
HERG K + Channel	E-4031	Zhou *et al*. (1999)
α-Galactosidase A (Fabry's Disease)	1-Deoxy-galactonojirimycin	Fan *et al*. (1999)
V2 Vasopressin receptor	SR121463, VPA 985	Morello *et al*. (2000)
Tyrosinase	DOPA, tyrosine	Halaban *et al*. (2001)
SUR1 (ABCC8)	Diazoxide, sulfonylureas	Partridge *et al*. (2001); Yan *et al*. (2004)
CFTR (ABCC7)	Benzo(c)quinolizinium compounds, doxorubicin, sildenafil, CFcor-325	Dormer *et al*. (2001, 2005); Loo *et al*. (2005); Maitra *et al*. (2001)
Anti-phosphocholine antibody	*p*-Nitrophenylphosphocholine	Wiens *et al*. (2001)
δ Opioid receptor	Naltrexone	Petaja-Repo *et al*. (2002)
Copper ATPase	Copper	Kim *et al*. (2002)
GnRH receptor	GnRH peptidomimetic antagonists	Janovick *et al*. (2002)
Cdr1p ABC transporter	Cycloheximide	Shukla *et al*. (2003)
Rhodopsin	Retinoids	Noorwez *et al*. (2003)
BCRP (ABCG2)	Mitoxantrone	Polgar *et al*. (2004)
MRP1 (ABCC1)	Glutathione	Buyse *et al*. (2004)
β-Hexosaminidae	*N*-Acetyglucoasamine-thazoline	Tropak *et al*. (2004)
Dopamine D4 receptor	Dopamine	Van Craenenbroeck *et al*. (2005)
Kainate receptors	Kainate, glutamate	Valluru *et al*. (2005)

Future studies on drug rescue of CFTR processing mutants will need to address the following questions:

1. Does CF_{cor}-325 interact directly with CFTR or indirectly affects a process involved with CFTR folding and/or trafficking?
2. Is the ΔF508 CFTR rescued by CF_{cor}-325 similar in structure to wild-type CFTR and have a similar half-life?
3. How toxic are these compounds? This will require animal studies.
4. How often would CF_{cor}-325 and its derivatives need to be administered to CF patients/animal to maintain enough CFTR at the cell surface?

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