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# Bithiazole Correctors Rescue CFTR Mutants by Two Different Mechanisms

Tip W. Loo, M. Claire Bartlett, and David M. Clarke*

Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Supporting Information

**ABSTRACT:** Better correctors are needed to repair cystic fibrosis transmembrane conductance regulator (CFTR) processing mutants that cause cystic fibrosis. Determining where the correctors bind to CFTR would aid in the development of new correctors. A recent study reported that the second nucleotide-binding domain (NBD2) was involved in binding of bithiazole correctors. Here, we show that bithiazole correctors could also rescue CFTR mutants that lacked NBD2. These results suggest that bithiazoles rescue CFTR mutants by two different mechanisms.

The cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) is an ATP-binding cassette (ABC) protein that acts as a cAMP-regulated chloride channel. It is a single polypeptide with five distinct domains: two nucleotide-binding domains (NBDs), two transmembrane domains (TMDs), and a regulatory (R) domain. CFTR located on the apical surface of epithelial cells that line lung airways and ducts of various glands (reviewed in [1]).

Cystic fibrosis (CF) is a genetic disease caused by mutations in the CFTR gene that affect function or synthesis.[2] The most common defect is deletion of Phe508 (ΔF508 CFTR) in the first nucleotide-binding domain (NBD1). ΔF508 is a processing mutation that inhibits folding in the endoplasmic reticulum and trafficking to the cell surface.[3] In addition, many of the rare CF mutants also contain processing mutations that inhibit maturation and trafficking to the cell surface.[4,5] The lack of chloride channel activity in CF patients due to defects in CFTR leads to recurrent lung infections and eventual lung failure.[6]

Processing mutants of CFTR's sister protein, the P-glycoprotein (P-gp) drug pump, can be specifically repaired by a drug rescue approach.[7] The mechanism of P-gp drug rescue is one in which binding of drug substrates to the TMDs promotes packing of the TM segments.[8] Because CFTR and P-gp are predicted to be structurally similar,[9] it may be possible to specifically repair CFTR processing mutants by a direct drug rescue approach using small molecules. Evidence that CFTR processing mutants could be repaired by a direct rescue approach is the observation that many second-site suppressors can rescue ΔF508 CFTR[10,11] and other CFTR processing mutants.[12] Potential advantages of a direct rescue approach are that expression of proteins involved in other metabolic pathways would not be altered and rescue would be less affected by differences in cellular folding environments in different tissues or in different disease states. Intensive efforts have been made to screen chemical libraries for compounds (called correctors) that could rescue ΔF508 CFTR. Unfortunately, rescue by correctors identified to date appears to be too weak for effective therapy.[13,14]

VX-809[15] and bithiazoles such as 4a[16] are the most promising specific correctors identified to date. VX-809[15] and 4a[16] appeared to be specific because they did not promote maturation of the hERG $K^+$ channel or dopamine receptor processing mutants. Most other correctors appeared to be nonspecific as they promoted maturation of P-gp processing mutants.[9]

Because VX-809 was the most effective corrector identified to date, it was tested in a clinical trial as a therapy for CF. Unfortunately, it was found that VX-809 caused only a small increase in sweat conductance, and no increases in the levels of mature ΔF508 CFTR were observed in rectal biopsies.[17] Therefore, a priority in the development of a CF therapy is to increase the maturation efficiency of CFTR processing mutants. To increase the maturation efficiency of CFTR processing mutants, it will be necessary to determine the mechanism of rescue of VX-809 and bithiazole-type correctors.

VX-809 appears to rescue CFTR processing mutants through direct interactions with TMD1.[12,18] Bithiazoles, however, appear to rescue CFTR processing mutants by a different mechanism because bithiazoles have an additive effect on maturation when used in combination with VX-809.[15] Identification of the bithiazole rescue site in CFTR is controversial as there is evidence that these compounds bind to NBD2[18] or to the TMDs.[9]

Evidence that bithiazoles interact with the TMDs was that 4a inhibited cross-linking between cysteines introduced into TMD1 and TMD2[19] and that 4a appears to stabilize TMD2.[20] In addition, it was found that bithiazoles enhanced core glycosylation of a truncation mutant that contained only the TMDs.[19] By contrast, it was recently reported that bithiazoles must interact with NBD2 because ΔF508 CFTR missing NBD2 was not rescued with bithiazoles and *in silico* docking predicted the presence of a bithiazole-binding site in NBD2.[18]

To test whether rescue of other CFTR processing mutants with bithiazoles (see Figure 1 for structures) was mediated by the TMDs or NBD2, we tested if bithiazoles could rescue full-length or ΔNBD2 CFTR mutants that had processing mutations in the TMDs such as G126D in TM2,[21] V232D in

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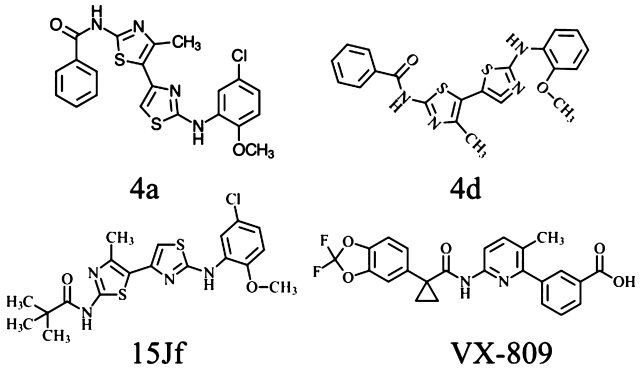


**Figure 1.** Structures of correctors.

TM4,[20] F337R in TM6,[12] and S1141R in TM12 (see Figure 2A). Mutants G126D and V232D are naturally occurring CF mutants, whereas F337R and S1141R mutants were constructed to map the orientation of the TM segments.[12] The mutants were expressed for 18 h with or without VX-809 or the 4a, 4d, or 15Jf bithiazole correctors.

Whole cell extracts were subjected to immunoblot analysis. Figure 2B shows that full-length and ΔNBD2 forms of G126D, V232D, and S1141R could be efficiently rescued with all the correctors such that the mature protein was the major product (Figure 2C,D). By contrast, only VX-809 promoted maturation of mutant F337R (Figure 2B). The bithiazoles did not promote maturation of the F337R ΔNBD2 or full-length F337R CFTR (Figure 2C,D).

The results suggest that bithiazoles rescue CFTR processing mutants by at least two different mechanisms. One mechanism involves binding of bithiazoles to a predicted bithiazole-binding site in NBD2.[18] For example, it was observed that removal of NBD2 from ΔF508 CFTR abolished rescue with a bithiazole (4a) but not VX-809.

It should be noted, however, that the ΔNBD2 CFTR used in this study was different from that used by Okiyoneda et al.[18] The ΔNBD2 CFTR constructs in our study did not contain the G550E, R553Q, R555K, and F494N mutations or the three hemagglutinin tags in the fourth extracellular loop that could influence CFTR corrector interactions.

The second bithiazole rescue mechanism appears to involve the TMDs because removal of NBD2 had little effect on bithiazole rescue of mutants G126D, V232D, and S1141R and mutation F337R in TM6 inhibited rescue of full-length CFTR with bithiazoles. Other pieces of evidence suggesting the bithiazoles affect folding of the TMDs were the observations that 4a blocked cross-linking between cysteines introduced into TM segments 6 and 12[19] and 15Jf promoted core glycosylation of a CFTR truncation mutant consisting of TMD1 and TMD2.[9] In addition, it was reported that 4a appeared to stabilize TMD2.[20]

VX-809 and bithiazoles appear to interact at different sites in the TMDs as the F337R mutant only prevented rescue with bithiazoles. These results could explain why VX-809 and bithiazoles have an additive effect on CFTR maturation.[15]

In summary, it appears that bithiazole correctors conduct rescues by at least two different mechanisms. One mechanism was proposed to target NBD2 misassembly.[18] We propose that the second mechanism is to promote packing of the TM segments. We predict that the CFTR TMDs are attractive targets for correctors because there is evidence that both bithiazoles and VX-809[12] can promote maturation through

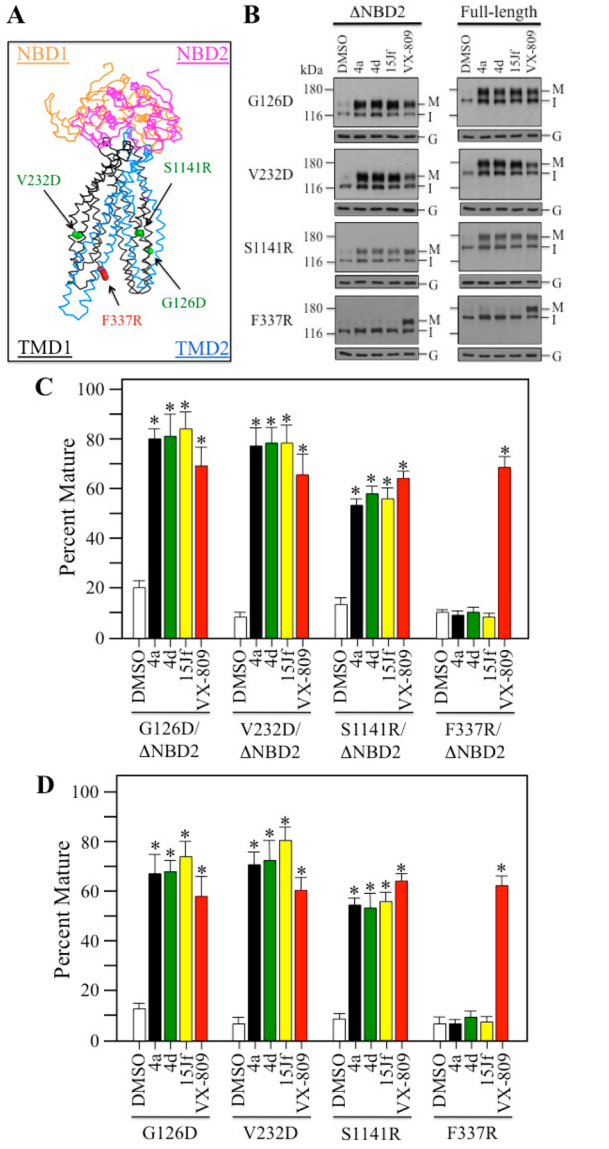


**Figure 2.** (A) Location of processing mutations in CFTR. (B) Effect of correctors on maturation of full-length and ΔNBD2 processing mutants. The positions of mature (M) and immature (I) CFTR and GAPDH (G) are shown. (C and D) Amounts of mature protein in ΔNBD2 and full-length CFTR processing mutants. An asterisk indicates a significant increase in the amount of mature protein compared to that without (DMSO) corrector.

interactions with the TMDs. Determining the mechanisms and targets of CFTR correctors might provide important insight into strategies for rescuing other misfolded proteins. It was recently shown that some CFTR correctors could also rescue trafficking mutants of arginine vasopressin receptor 2, hERG and SUR1.[22]

## ■ ASSOCIATED CONTENT

### Supporting Information

Details of experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.


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## AUTHOR INFORMATION


### Corresponding Author

*Department of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada. E-mail: david.clarke@utoronto.ca. Telephone and fax: (416) 978-1105.



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### Notes

The authors declare no competing financial interest.

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