Rescuing Trafficking Mutants of the ATP-binding Cassette Protein, ABCA4, with Small Molecule Correctors as a Treatment for Stargardt Eye Disease*

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Background: Mutations in nucleotide binding domain 1 of ABCA4 cause Stargardt Disease.

Results: Correctors rescue trafficking of NBD1 mutants by altering a proteostatic network of quality control proteins.

Conclusion: Rescue of trafficking ABCA4 mutants can be accomplished by correctors similar to CFTR.

Significance: There is currently no treatment for Stargardt macular degeneration.

Stargardt disease is the most common form of early onset macular degeneration. Mutations in ABCA4, a member of the ATP-binding cassette (ABC) family, are associated with Stargardt disease. Here, we have examined two disease-causing mutations in the NBD1 region of ABCA4, R1108C, and R1129C, which occur within regions of high similarity with CFTR, another ABC transporter gene, which is associated with cystic fibrosis. We show that R1108C and R1129C are both temperature-sensitive processing mutants that engage the cellular quality control mechanism and show a strong interaction with the chaperone Hsp 27. Both mutant proteins also interact with HDCAC6 and are degraded in the aggresome. We also demonstrate that novel corrector compounds that are being tested as treatment for cystic fibrosis, such as VX-809, can rescue the processing of the ABCA4 mutants, particularly their expression at the cell surface, and can reduce their binding to HDAC6. Thus, our data suggest that VX-809 can potentially be developed as a new therapy for Stargardt disease, for which there is currently no treatment.

Stargardt macular degeneration is the most common form of early onset macular degeneration, causing poor visual outcome (32). The prevalence is ~1 in 10,000 (23). Individuals with this disorder suffer from a loss of central vision and impaired dark adaptation due to progressive accumulation of lipofuscin that results in dysfunction of the retinal pigmented epithelium (RPE) and photoreceptors (24). Mutations in ABCA4 are associated with Stargardt disease (17). In addition to being causative for Stargardt disease, ABCA4 mutations are associated with severe forms of retinal degeneration, such as recessive cone-rod dystrophy and recessive retinitis pigmentosa (24). Importantly, mutations in ABCA4 present in heterozygotes may be the cause of adult onset macular degeneration in ~16% of all cases (5, 33).

ABCA4 is a member of the ATP-binding cassette (ABC) proteins. ABC proteins are divided into seven subfamilies (15), with common motifs that include two sets of transmembrane domains (TMD) and two nucleotide-binding domains (NBD). The functional unit is either contained in one protein comprised of the four domains encoded by one gene or comprised of two or more proteins each containing the individual domains that assemble themselves post-translationally. ABCA subfamily members typically are lipid transport proteins with all the ABC protein motifs combined in one protein. They have two characteristically large glycosylated extracellular loops (1 and 4) in TMD1 and TMD2 (3), which contribute to their rather large molecular weight.

ABCA4, also known as the Rim protein or ABCR, is a 2,273-amino acid protein expressed in outer segments of the disk in rod and cone photoreceptors of the vertebrate retina (21). ABCA4 has an extended C-terminal tail containing a VFVNFA sequence that is also present in ABCA1, but not found in other members of the family. Many ABC proteins are efflux pumps, but ABCA4 is an ATP-dependent import pump whose major substrate is N-retinylidene-phosphatidylethanolamine (N-retinylidene-PE) (28). In the daylight, all-trans-retinal (ATR) disassociates from rhodopsin, where it binds to PE, creating N-retinylidene-PE in the RPE. ABCA4 transports N-retinylidene-PE to the cytosolic leaflet of the cell membrane, where it dissociates into ATR and PE. ATR within the RPE cell is reduced to all-trans retinol by retinol dehydrogenase, the latter entering the visual cycle for conversion to 11-cis retinal (12). When ABCA4 is mutated, transport is compromised, allowing for the accumulation of ATR within the disc lumens and gradually creating diethrinoid-pyridinium-ethanolamine (A2E) and -retinal dimer (RALdi). The latter are the main components of lipofuscin (23). This scenario is clearly evident in the abca4 knock-out mouse, in which there is a progressive, light-depen-

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3 The abbreviations used are: RPE, retinal pigmented epithelium; ERAD, endoplasmic reticulum-associated protein degradation; ABC, ATP-binding cassette; TMD, transmembrane domain; PE, phosphatidylethanolamine; ATR, all-trans retinal; CFTR, transmembrane conductance regulator; VCP, valosin-containing protein.

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dent increase in ATR, N-retinylidene-PE, RALdi, and A2E, leading to the accumulation of lipofuscin products in the RPE (38).

More than 500 mutations in the ABCA4 gene have been linked with retinal disease, including Stargardt macular degeneration (24), which leads to progressive vision loss that invariably ends in legal blindness. There is no known treatment for this disease. Clearly, restoring ABCA4 function would be the best way to treat the disease, either with gene therapy or by repairing the mutant protein. Success in the realm of protein repair has occurred for the transmembrane conductance regulator (CFTR) (36), which belongs to the ABCB family and functions as a chloride channel (4). Over 1,000 mutations in CFTR lead to cystic fibrosis (CF), an autosomal recessive disease associated with defective fluid transport across several mucosal membranes throughout the body (31). Even though CFTR is approximately half the size of ABCA4 and has a unique regulatory domain located between NBD1 and TMD2, both ABC transporters share sequence homology. Fig. 1 shows the sequence homology within NBD1 of CFTR (Fig. 1A) and ABCA4 (Fig. 1B). Both have classical ABC transporter motifs, including the Walker A domain, with a conserved leucine residue, the Q loop, the LSGGQ signature sequence, and the Walker B domain with conserved Asp and Ala residues. The conserved phenylalanine at position 508 of the CFTR gene (1026 in ABCA4) is deleted in the majority of patients with CF (ΔF508-CFTR), causing severe disease (30).

Knowledge of the genetic and molecular basis for CF, especially with regard to the most common mutation, ΔF508-CFTR, has led to the identification and study of “correctors” that rescue mutant trafficking and function, such as VX-809, which has been shown to be safe with biological activity in a Phase IIa clinical trial (11). Given the conserved motifs and sequence similarity within the NBD1 domain between CFTR and ABCA4 (Fig. 1), we hypothesized that strategies currently being applied to rescue ΔF508-CFTR could be applied to similar mutations in ABCA4. The extended application of correctors is in particular, attractive as a novel therapy for Stargardt disease, which has no known treatment, in carefully selected mutations of ABCA4 that are similar to those in CFTR (Fig. 1) because of VX-809’s safety profile.

Materials and Methods

Generation of Stably Transfected HEK 293 Cell Lines—Flp-In human embryonic kidney (HEK) 293 cells (Cat. CRL-1573, Life Technologies) were cultured in Dulbecco’s minimal essential medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (FBS), with penicillin (100 units/ml), streptomycin (100 μg/ml), and Zeocin (100 μg/ml) at 37 °C in 5% CO2. After transfection, the medium was replaced with DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and hygromycin (100 μg/ml). Hygromycin-resistant foci were isolated, expanded, and then analyzed for expression of ABCA4, R1108C, or R1129C by Western blotting.

Biotinylation—HEK 293 cells stably expressing ABCA4, R1108C, or R1129C were exposed to sulfo-NHS-SS-biotin (Thermo Scientific) for 30 min on ice, rinsed three times with glycine quenching buffer (200 mM glycine and 25 mM Tris/HCl, pH 8.0, in DPBS with calcium and magnesium), and solubilized in lysis buffer (150 mM NaCl, 50 mM Tris/HCl, 1% Nonidet P-40, and protease inhibitors). To extract proteins, the lysates were centrifuged at 30 min at 4 °C. The resulting lysates were centrifuged at 13,200 rpm for 15 min at 4 °C, and total cellular protein content was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad). The supernatants were incubated with NeutrAvidin Plus UltraLink Resin (Thermo Scientific) for 1 h at 4 °C (40 μg of protein/l μl of beads). After brief centrifugation, the supernatant was removed, and the beads were washed five times with lysis buffer. After the last washing step, the beads were mixed with 2× Laemmli Sample Buffer with β-mercaptoethanol (Bio-Rad) to elute the adsorbed proteins.

Western Blotting—Protein samples were electrophoresed on SDS/PAGE (7.5% gels) and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dried skimmed milk in TBS Tris-buffered saline (TTBS: 50 mM Tris and 150 mM NaCl, pH 8.0, with 0.05% Tween 20) for 1 h, then incubated overnight at 4 °C with an anti-ABCA4 antibody (sc-58758, Santa Cruz Biotechnology). Ezrin (sc-57858, Santa Cruz Biotechnology) detection was used as a loading control. The blots were then washed and incubated with sheep anti-mouse IgG peroxidase linked antibody (GE Healthcare). Immunoreactive bands were visualized by SuperSignal West Dura substrate (Thermo Scientific) using a Fuji Film LAS-4000 Plus system with a cooled CCD camera. Image Gauge version 3.2 software (Fuji Film) was used for quantification of the bands. Background was determined in the vicinity of each of the band quantified and the background values subtracted.

Immunoprecipitation—Proteins were extracted, and protein concentrations were measured as described above. Then 2000 μg of protein lysate were rotated with 80 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) and 4 μg of ABCA4 antibody for 4 h at 4 °C. The A/G beads were centrifuged at 2000 rpm for 2 min on a high-speed tabletop centrifuge, the supernatants were discarded, and the beads were washed four times with lysis buffer plus protease inhibitors. 2× sample buffer with β-mercaptoethanol was added with the beads at a 1:1 ratio. Samples were used for Western blotting as described above,
and for co-immunoprecipitation (co-IP), antibodies specific for the protein of interest were applied.

Small-molecule Correctors and Inhibitors—Small-molecule correctors C3, C4, C18, and 809 obtained from the CFFT Panel library were tested alone or in combinations of two. The effect of proteasome, aggresome, and autophagosome inhibitors (MG132, tubacin, and bafilomycin A1, respectively) on wt ABCA4, R1108C, and R1129C mutations was also studied. Dose-response experiments with incremental doses were performed, using 1, 5, 10, and 20 \( \mu M \) for the three correctors and tubacin; 1, 10, 50, and 100 \( \mu M \) for bafilomycin A; and 1, 3, 5, and 10 \( \mu M \) for MG132.

Results

Processing Mutants of ABCA4—Genetic mutations that cause human disease have different effects on the proteins that they encode (6). A subset of the mutants results in misfolded proteins that are prematurely targeted for degradation by the ER-associated quality control pathways (6). For this study, we focused on two disease-causing mutations in ABCA4, R1108C, and R1129C, both of which are known to be less abundant than the wt (35). The mutations are in the NBD1 domain of ABCA4, just beyond the Walker B region (Fig. 1A). Confirming previous reports, we also found that both mutants were less abundant than wt ABCA4 (Fig. 1B). We then asked whether these mutant proteins were recognized by the cell’s quality control mechanism and rapidly degraded. As a first step, we grew the cells at reduced temperature to determine whether they were temperature-sensitive. Fig. 2 shows that the steady-state protein levels of R1108C and R1129C were dramatically increased when cells were grown at reduced temperature. This result is in sharp contrast to the effects of proteasomal and lysosomal inhibitors had similar effects on ABCA4. Given that tubacin had the greatest effect, these data suggest that the two mutants are degraded via the aggresome.
R1108C and R1129C Have Heightened Interactions with Proteins Associated with Their Degradation—Folding of complex proteins such as ABCA4 relies on a series of chaperones. When complex proteins fail to fold properly, chaperones assist the cell in recognizing mutant proteins for degradation (6). To determine how ABCA4 interacts with chaperones, we conducted co-IP studies with four chaperones: Hsp27, 40, 70, and 90. Hsp 70 and its co-chaperone Hsp 40 are key players in protein folding (20). Hsp 90 functions downstream of Hsp 70 and plays a protective role by allowing the hydrophobic domains of certain proteins to fold properly (14). Hsp 27 (HspB1), a member of the small heat shock protein family, is thought to guard against protein aggregation during stress (20). In addition to the chaperones, we also determined the binding of ABCA4 and its mutants to valosin-containing protein (VCP) and the histone deacetylase, HDAC6, which translocate mutant proteins to the proteasome (8) and aggresome (19), respectively. Among the results of all experiments performed, two observations stood out: First, Hsp 27 showed a dramatic decrease in the total amount present in cells stably expressing the mutants, when compared with the cells expressing wt ABCA4 (Fig. 7). Despite the decrease in Hsp 27 in the total lysate, the amount co-immunoprecipitated remained relatively constant, leading to a 6-fold increase in the protein ratio in the co-immunoprecipitate/total lysate (co-IP/TL), suggestive of increased binding to the mutants. Second, HDAC6 demonstrated an increase in the amount co-immunoprecipitated as compared with the controls. No significant differences were seen in the co-IP/TL ratio for the other chaperones tested or for VCP (Fig. 8). The increase in binding of HDAC6 to the mutants and the lack of an effect on

**FIGURE 2. Temperature sensitivity of wt ABCA4, R1108C, and R1129C.** HEK-293 cell lines stably expressing wild type (wt) ABCA4, R1108C, or R1129C were grown continuously at 37 °C or 27 °C. Growing cells with R1108C or R1129C at reduced temperature significantly increased the amount of ABCA4 expressed. Curiously, when wt ABCA4 was grown at low temperature, the expression was reduced. *, p < 0.05; **, p < 0.01; ***, p < 0.0001). n = 3. Ezrin was used as the loading control.

**FIGURE 3. Disappearance of protein from wt ABCA4 and from R1108C and R1129C mutants.** HEK-293 cell lines stably expressing wt ABCA4, R1108C, or R1129C were treated with cycloheximide (25 μg/ml) and harvested at 1, 2, 4, 6, or 8 h. The protein expressed from mutations R1108C and R1129C disappeared much faster than that from wt ABCA4 when protein translation was inhibited with cycloheximide, indicating that they are much less stable. C: control. n = 3. Ezrin was used as the loading control.
VCP binding is consistent with the data presented earlier, showing that R1108C and R1129C are preferentially degraded in the aggresome.

**ABCA4 Mutants Are Rescued by the Same Correctors that Rescue ΔF508-CFTR**—As Fig. 1A shows, there is ~45% similarity in the NBD1 motifs of ABCA4 and CFTR. There are several instances in which amino acids are identical or the substitutions are conserved. For example, the phenylalanine that is missing from ΔF508-CFTR is in an identical position in ABCA4. Given this degree of similarity, we asked whether correctors that rescue ΔF508-CFTR could also rescue ABCA4. A number of small molecules have been identified that rescue ΔF508-CFTR. We tested four of them: C3, C18, and VX-809, discovered by Vertex Pharmaceuticals (36), and C4, discovered by Verkman (27) and collaborators. It is important to note that VX-809, under the trade name Lumacaftor, was recently shown to be safe with biological activity in a phase IIa clinical trial (11). These correctors have recently been characterized into different classes, depending upon their mode of action. C3, C18, and VX-809, all in class I, are thought to stabilize NBD1-TMD1/2 interfaces. C4 has been assigned to class II, the compounds that restore NBD2 or its interfaces (26). We applied C4 to both the wt and mutant ABCA4 and noted a maximum response of ~1.5-fold increased protein expression for both wt and mutant

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**FIGURE 4. Proteasomal degradation pathway.** HEK-293 cell lines stably expressing wt ABCA4, R1108C, or R1129C were treated for 16 h with increasing doses of proteasome inhibitor (MG-132). Data are normalized to 0 μM control. *, p < 0.05. n = 3–4. Ezrin was used as the loading control.

**FIGURE 5. Aggresomal degradation pathway.** HEK-293 cell lines stably expressing wt ABCA4, R1108C, or R1129C were treated for 16 h with increasing doses of the aggresome inhibitor tubacin. Data are normalized to 0 μM control. *, p < 0.05. n = 3–4. Ezrin was used as the loading control.

**FIGURE 6. Lysosomal degradation pathway.** HEK-293 cell lines stably expressing wt ABCA4, R1108C, or R1129C were treated for 16 h with increasing doses of the proton pump inhibitor of lysosome degradation bafilomycin A. Data are normalized to 0 μM control. *, p < 0.05. n = 3–4. Ezrin was used as the loading control.
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ABCA4 (Fig. 9). We next applied C3 and noted a more robust response, a nearly 2-fold increase, with the R1129C mutation showing the greatest response (Fig. 10). The greater response from C3 than C4 suggests that Class 1 correctors might work better than Class II for these ABCA4 mutations. For the ΔF508 mutation in CFTR, applying both correctors has been shown to result in greater rescue (7). However, application of C3 + C4 did not produce a greater response than that of either corrector applied individually (Fig. 11). Like C3, another Class I corrector, C18, also produced a robust response, achieving a ~2-fold maximum increase in protein expression with the R1108C and R1129C mutants (Fig. 12A). This result is important because C18 is closely related to VX-809 (Fig. 12B) (16), which, as mentioned earlier, is already in clinical trials to treat CF (11). Combinations of C18 + C4 or C18 + C3 (Figs. 13 and 14) appeared to be less effective than when the correctors are used individually. Thus, it is clear that C18 added alone gives the best effect (Fig. 12A).

As mentioned above, VX-809 is important because it has already passed many of the regulatory hurdles involved in

FIGURE 7. Quality control protein interactions with ABCA4, R1108C, and R1129C: total lysate (TL) and co-immunoprecipitate (IP). Samples were used for Western blot analysis and blotted with antibodies detecting Hsp 27, HDAC6, or ABCA4. As a control, ABCA4 was immunoprecipitated with anti-ABCA4 antibody in the wt, R1108C, and R1129C cell lines. The data show that when both mutants were expressed in the HEK cell line, there was significantly less Hsp27 in the total lysate. Binding of Hsp 27 to the mutants was approximately 4–5-fold greater than to wt ABCA4. Also, the binding of HDAC6 with the mutation was increased by ~1.5–1.7-fold. Data are normalized to wt. *, p < 0.05; **, p < 0.01. n = 5 independent experiments. The magnitude of binding was assessed using the co-IP/TL ratio.

FIGURE 8. Binding of wt and mutant ABCA4 to Hsp 90, 40, 70, and VCP: total lysate (TL) and co-immunoprecipitate (IP). Samples were used for Western blot analysis and blotted to detect Hsp 90, 40, 70, and VCP. No significant differences in the binding to ABCA4 were noted here among the wt and the mutants. These data indicate that the mutants do not engage these quality-control proteins to any greater extent than does the wt ABCA4. Normalized to wt. *, p < 0.05; **, p < 0.01. n = 5. The magnitude of binding was assessed using the co-IP/TL ratio.
approval for patient use (11). Fig. 12B shows that VX-809 was able to rescue both R1108C and R1129C. Significantly, its greatest effect was to increase the surface expression of both mutants (Fig. 15). Given that a mutant ABCA4 protein must pass several quality-control checkpoints to reach the cell surface, the observation that VX-809 increases cell-surface expression indicates that the corrector has significantly rescued the stability of the mutants.

VX-809 Reduces the Interactions of R1108C and R1129C with Proteins Associated with Their Degradation—Figs. 16 to 18 show the effect of correctors on the binding of wt ABCA4 to HDAC6 and HSP 27. Both correctors caused either a further increase or no change in the binding of wt or mutant protein to HSP27. Interestingly, both correctors reduced the binding of wt (Fig. 16), R1108C (Fig. 17), and R1129C (Fig. 18) to HDAC6. The correctors C18 and VX-809 both increased the binding of Hsp 27 to wt ABCA4 (Fig. 16). In contrast, the binding of Hsp 27 to the mutants was not influenced by the correctors (Figs. 17 and 18).

Discussion

Here we have shown that two disease-causing mutations in abca4 produce proteins that display the classic characteristics of processing mutants, including reduced state-state protein levels, temperature-sensitivity, and rapid protein degradation. There are two pathways whereby misfolded proteins can be degraded. The most common pathway is through the process known as endoplasmic reticulum-associated degradation (ERAD). As the cell’s quality-control mechanism, ERAD operates by recognizing misfolded proteins early in their biosynthesis, ubiquitinating them and moving them to the proteasome through the action of VCP and its interacting proteins (8). VCP, also known as p97, plays a major role in the extraction of misfolded proteins from the ER membrane, their transport to the proteasome, and the trimming of ubiquitin chains to facilitate proteasomal degradation (8). We have now made the interesting observation that VCP binds to both wt and mutant ABCA4. It is not surprising that VCP binds to wt ABCA4.
because it is well known that a significant portion of the individual wt proteins made by the cell are defective and must be degraded (37). What is interesting is that the VCP binding to the mutants was not significantly greater than that to wt ABCA4, suggesting against an enhanced role for VCP in the degradation of the mutants. Consistent with this finding are our results with MG-132, a nonspecific inhibitor of proteasomal degradation (9). Application of MG-132 caused a noticeable decrease in mutant protein levels, which suggests an alternate mechanism of mutant protein degradation in the aggresome.

The aggresome collects aggregates of misfolded proteins, especially during proteasome blockade. Aggregated, misfolded proteins are collected by microtubule networks and transported to the microtubule organizing center and degraded in bulk by autophagy (39). HDAC6 is well known to transport misfolded protein aggregates to the aggresome (39). Two sets of
observations from our experiments here support our contention that R1108C and R1129C are degraded by the aggresome. First, inhibition of HDAC6 caused a 2-fold increase in mutant protein abundance and restored mutant protein levels close to wt ABCA4. Secondly, HDAC6 displayed increased binding to mutants relative to wt ABCA4. A similar duality in the degradation of H9004F508-CFTR also occurs. Because of its tendency to aggregate, H9004F508-CFTR is degraded both by the proteasome and aggresome (18). This similarity in how mutant ABCA4 and H9004F508-CFTR are recognized by the cellular quality control mechanism is not surprising, given the degree of similarity between the two proteins, particularly within the NBDs.

Chaperone proteins are well known to help CFTR to fold properly and to target misfolded mutant CFTR for degradation (6). To shed light on the involvement of ABCA4 and its mutants with chaperones, we determined its binding to several heat shock proteins. Of the four chaperones tested, HSP 27 stood out. This chaperone bound avidly to both R1108C and R1129C. HSP27 is a member of the family of small heat shock proteins (13). Unlike larger chaperones such as HSP 70 and 90, which require ATP to function, HSP 27 does not. HSP 27 is also involved in the degradation of H508-CFTR (2) by ligating it with the small ubiquitin-like modifier SUMO-2 (1). Like ubiquitin, SUMO1 and 2/3 attach to proteins via specific ligases. Hsp 27 facilitates the attachment of SUMO-2 to CFTR. HSP 27 can distinguish between different folded states of proteins and prefers binding to conformations that are ultimately folded, rather than binding to totally denatured or wt proteins (13). Hsp 27 can form large oligomers that function as chaperones to prevent the aggregation of misfolded proteins (34). It can be phosphorylated at three serine residues, which can limit the degree of oligomerization and chaperone activity. Thus, enhanced HSP 27 binding to the ABCA4 mutants could
serve two roles: to aid in SUMO-2 targeting and to prevent aggregation.

High impact work on cystic fibrosis using high-throughput screening or structurally based drug design has led to substantial understanding of the impact of the missing phenylalanine at residue 508 of CFTR, which has resulted in the development of small-molecule correctors that can reverse the impact of this mutation. Basically, the F9004/H9004 F508-CFTR mutation induces two defects that must be rescued: 1) thermal instability of NBD1, and 2) improper domain-domain interactions, especially between NBD1 and the intracellular loops (22). Although the precise defects in the structure of R1108C and R1129C are not known, given the similarity in the way that ΔF508-CFTR and these ABCA4 mutants interact with the cellular quality-control mechanism, we hypothesized that correctors that could rescue ΔF508-CFTR might also rescue R1108C and R1129C. To test this hypothesis, we looked at correctors from two different classes, class I (C3, C18, and VX-809), which are thought to stabilize the NBD1-TMD1/2 interfaces; and class II (C4), which are thought to restore NBD2 or its interfaces (26). Our results indicate that class I correctors increased the protein processing of both ABCA4 mutants.
The response of class I correctors on ΔF508-CFTR is known to be biphasic with one phase involving rescue while higher doses are inhibitory (36). The reason for this biphasic response is unclear, but it is also evident in our data regarding ABCA4. As seen in Fig. 13 & 14, lower doses rescued the mutant proteins while higher corrector doses inhibited the rescue of mutant ABCA4. This biphasic response may explain why adding two correctors, C3 + C18, from the same class I diminished instead of enhanced the rescue. We were surprised that the combination of C4 + C18, from two different classes, did not enhance the recovery of mutant proteins. It is possible that we did not fully identify the optimal doses for combination treatment. With a proven safety profile in a phase IIa clinical trial to treat cystic fibrosis (11), VX-809 could potentially be used to treat Stargardt disease. Our results provide hope that one corrector may be sufficient to rescue certain ABCA4 mutants.

FIGURE 17. Effect of C18 and VX-809 on the binding of R1108C ABCA4 to Hsp 27 and HDAC6; total lysate (TL) and co-immunoprecipitate (IP). HEK 293 cell lines expressing R1108C were treated with C18 or VX-809, and ABCA4 was immunoprecipitated with anti-ABCA4 antibody. Western blotting was performed with anti-ABC4, anti-HDAC6, and anti-HSP27 antibodies. HDAC6 showed reduced binding after treatment with C18 or VX-809; HSP27 binding was increased slightly after treatment with C18 or VX-809. Data are normalized to untreated control values. *, p < 0.05; **, p < 0.01. n = 4. The magnitude of the binding was assessed using the co-IP/TL ratio.

FIGURE 18. Effect of C18 and VX-809 on the binding of wt and mutant R1129C to Hsp 27 and HDAC6; total lysate (TL) and co-immunoprecipitate (IP). HEK 293 cell lines expressing R1129C were treated with C18 or VX-809, and ABCA4 was immunoprecipitated with anti-ABCA4 antibody as a control. Western blotting was performed with anti-ABC4, anti-HDAC6, and anti-HSP27 antibodies. HDAC6 showed reduced binding after treatment with C18 or VX-809, but HSP27 binding was increased slightly after treatment with C18 or VX-809. Data are normalized to untreated control values. *, p < 0.05; **, p < 0.01. n = 4. The magnitude of the binding was assessed using the co-IP/TL ratio.

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As mentioned above, ABCA4 is a retinylidene-phosphatidylethanolamine transporter that facilitates the recycling of all-
trans retinal, which originates from photoreceptors following photoexcitation (28). The question here is whether the rescue of ABCA4 that we have demonstrated in our studies will have therapeutic potential. Although, VX-809 increases the total amount of ABCA4 protein, the major effect is to increase the surface expression of both mutants. Processing mutants such as ΔF508-CFTR have to pass several quality-control checkpoints before reaching their functional location (40). Thus, increased plasma membrane levels of both R1108C and R1129C suggests that the restoration of protein stability by VX-809 is sufficient to allow the mutant proteins to pass or avoid the checkpoints, and proceed to the plasma membrane. Thus, observation that VX-809 most likely by increasing the stability of the mutants and thereby reducing the binding of the mutants to HDAC6 enabled them to bypass HDAC6 mediated processing by the aggresome. VX-809 rescues not only CFTR processing but also chloride channel function (36), and as such we would expect ABCA4 function to also be maintained. Even though CFTR chloride channel function is restored in in vitro experiments, in clinical trials with VX-809, CF patients have required both a corrector and a potentiatior to observe clinical benefit (11). Thus, the question that could be raised here is the extent that VX-809 will rescue ABCA4 activity to have a clinical impact. Under in vitro conditions with ample protein in transfected cells, C18 and VX-809 easily restore CFTR trafficking and function (29). However, in vivo, CFTR is present at very low levels in the human lung, and thus, a single corrector may be inadequate because of protein levels below a threshold where rescue is possible rather than inherent transport function deficiencies of the mutant protein. In contrast, since it plays a key role in the light cycle, and is known to be abundantly expressed in the RPE, it of the mutant protein. In contrast, since it plays a key role in the

Conclusion

In the present study, we have applied a strong understanding of the structure-function relationships of ABCA4 and CFTR to show that mutations in abca4 that occur in regions of high similarity between the two proteins have very similar impact. We show that R1108C and R1129C in NBD1 are both temperature-sensitive processing mutants that engage the cellular quality control mechanism via a strong interaction with the chaperone Hsp 27. Both mutants also interact with HDCAC6 and are degraded in the aggresome. Finally, novel corrector compounds that are being applied to the treatment of cystic fibrosis can also rescue the processing of ABCA4 mutants, potentially fast-tracking the development of new therapies to treat Stargardt disease, for which there is currently no treatment.

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