Mapping the Binding Site of the Inhibitor Tariquidar That Stabilizes the First Transmembrane Domain of P-glycoprotein

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ABC (ATP-binding cassette) transporters are clinically important because drug pumps like P-glycoprotein (P-gp, ABCB1) confer multidrug resistance and mutant ABC proteins are responsible for many protein-folding diseases such as cystic fibrosis. Identification of the tariquidar-binding site has been the subject of intensive molecular modeling studies because it is the most potent inhibitor and corrector of P-gp. Tariquidar is a unique P-gp inhibitor because it locks the pump in a conformation that blocks drug efflux but activates ATPase activity. In silico docking studies have identified several potential tariquidar-binding sites. Here, we show through cross-linking studies that tariquidar most likely binds to sites within the transmembrane (TM) segments located in one wing or at the interface between the two wings (12 TM segments form 2 divergent wings). We then introduced arginine residues at all positions in the 12 TM segments (223 mutants) of P-gp. The rationale was that a charged residue in the drug-binding pocket would disrupt hydrophobic interaction with tariquidar and inhibit its ability to rescue processing mutants or stimulate ATPase activity. Arginines introduced at 30 positions significantly inhibited tariquidar rescue of a processing mutant and activation of ATPase activity. The results suggest that tariquidar binds to a site within the drug-binding pocket at the interface between the TM segments of both structural wings. Tariquidar differed from other drug substrates, however, as it stabilized the first TM domain. Stabilization of the first TM domain appears to be a key mechanism for high efficiency rescue of ABC processing mutants that cause disease.

Human ATP-binding cassette (ABC) transport proteins mediate the ATP-dependent flux of substrates across extra- or intracellular membranes including metabolic products, lipids, sterols, ions, and drugs. Multidrug resistance in cancer, cystic fibrosis (CF), gout, intrahepatic cholestasis types 2 and 3, Tangier disease, Dubin-Johnson syndrome, hyperinsulinimic hypoglycemia of infancy, pseudoxyanthoma elasticum, secretory diarrheas, gout, anemia, diabetes, and atherosclerosis are potentially lethal or debilitating conditions caused by mutations or altered activity of one of the 48 human ABC (ATP-binding cassette) proteins (1).

Considerable efforts have been made to achieve two clinical goals: 1) turn off ABC drug pumps such P-gp (ABCB1), BCRP (ABCG2), and MRP1 (ABCC1) to prevent multidrug resistance; and 2) repair folding mutants of CFTR (ABCC7; cystic fibrosis), BCRP (ABCG2; gout), ABCB4/ABCB11 (progressive familial intrahepatic cholestasis), ABCA1 (Tangier disease), ABC2 (Dubin-Johnson syndrome), ABCC6 (pseudoxyanthoma elasticum), and ABCC8 (hyperinsulinimic hypoglycemia of infancy) using small molecules called correctors or pharmacological chaperones. Large scale screening efforts have been promising but most compounds identified to date have been poor therapeutic candidates as they only caused partial inhibition of the pump or rescue of misfolded ABC proteins.

Studying P-gp interaction with tariquidar is important for understanding how to turn off drug pumps or rescue misfolded mutants. Tariquidar is different from previous inhibitors because it is not transported by P-gp but binds to the protein to activate ATPase activity (2). Although tariquidar is the most effective inhibitor identified to date, it still needs to be improved as clinical trials suggest that inhibition of transport is not complete (3). It appears that P-gp needs to be almost completely inactivated to improve therapy because it works so rapidly and with such high capacity that it can still prevent entry of substrates even when most of the pumps are turned off (4).

Tariquidar is also the most potent P-gp corrector as processing mutants could be rescued by carrying out expression in 100 nM tariquidar (2). Rescue of P-gp processing mutants with other drug substrates (5) or rescue of other ABC proteins with correctors requires at least 10-fold higher concentrations (6).

To understand P-gp/tariquidar interactions, many in silico docking studies have been performed to identify the drug-binding sites (7–12). In retrospect, some of the earlier docking and molecular dynamic studies done with human P-gp homology models were suboptimal because they were based on the crystal structures of ABC transporters from Staphylococcus aureus bacteria (Sav1866) (11), Caenorhabditis elegans (12), or an earlier mouse structure (13). There is low sequence homology between Sav1866 and human P-gp in the TMDs, the structure...
of TM10 in C. elegans P-gp is undefined, whereas the earlier P-gp structure from mouse was subsequently found to contain quite a few errors (14–17). In silico docking (8, 9) and molecular dynamics studies (9) have recently been done with homology models of human P-gp based on the corrected crystal structures of P-gp from mouse (16). For example, McCormick et al. (9) performed molecular dynamic simulations of human P-gp to show transport of two different substrates through the plane of the membrane. By contrast, tariquidar did not show this movement but stabilized P-gp in an outward open conformation. They also identified three potential tariquidar-binding sites. Therefore, one of our goals was to biochemically test these predictions.

We previously used alanine scanning mutagenesis to map the locations of substrate-binding sites in a membrane transport protein (SERCA1 Ca-ATPase) (18). A problem with using alanine-scanning mutagenesis to map the location of drug-binding sites in P-gp was that introduction of a small side chain in the drug-binding pocket caused little detectable effect on binding of relatively large drug substrates (19, 20). Another problem is that a small change to alter the hydrophobicity of a side chain simply changes the substrate specificity of P-gp (21).

Here, we used cross-linking protection assays and arginine mutagenesis of residues within the 12 TM segments to test for residues close to or within the tariquidar-binding site. The rationale for arginine mutagenesis was that insertion of a bulky charged side chain into a tariquidar-binding site would inhibit tariquidar rescue of processing mutants and tariquidar-stimulated ATPase activity. Our results suggest that tariquidared binds to a site within the drug-binding pocket because 30 arginines introduced into the TM segments disrupted both tariquidar rescue of processing mutants and tariquidar-stimulated ATPase activity. Unlike other drug substrates, tariquidar promoted maturation and stabilized the first transmembrane domain (TMD1). Stabilization of TMD1 may be an important mechanism in rescuing misfolded ABC proteins because a similar mechanism appears to be involved in the rescue of misprocessed CFTR protein by the corrector VX-809 (22).

**Experimental Procedures**

**Construction of Mutants**—Mutations were introduced into the wild-type, Cys-less, or G251V P-gp cDNAs (residues 1–1280) containing the A52-epitope or 10-histidine tags (23) of the wild-type, Cys-less, or G251V P-gp cDNAs (residues 1–1280) containing the A52-epitope or 10-histidine tags (23) of the wild-type, Cys-less, or G251V P-gp cDNAs (residues 1–1280) containing the A52-epitope or 10-histidine tags (23) of the wild-type, Cys-less, or G251V P-gp cDNAs (residues 1–1280) containing the A52-epitope or 10-histidine tags (23). For expression and maturation of G251V Arginine Mutants—The G251V mutant cDNAs were transiently expressed in HEK 293 cells by a calcium phosphate precipitation approach as described previously (28). Briefly, 10 μl of 2.5 mM CaCl2 was added to 90 μl of H2O containing 2 μg of DNA followed by addition of 100 μl of BES solution (50 mM BES, 280 mM NaCl, and 1.5 mM Na2HPO4, pH 6.96). After 10 min at room temperature, 4 ml of HEK 293 cells (about 100,000 cells/ml) in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (supplemented with nonessential amino acids, 4 mM t-glutamine, 10 IU/ml of penicillin, 10 μg/ml of streptomycin, and 1% (v/v) bovine calf serum) was added and 1.5 ml of the mixtures were added to duplicate well of 6-well culture plates. After 4 h at 37 °C, the medium was replaced with fresh medium with or without 0.5 μM tariquidar (UHN (Shanghai) Research & Development Co., Toronto, ON). About 16 h later, the cells were harvested, washed with PBS, and cell pellets were suspended in 150 μl of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol containing 25 mM EDTA). Samples were applied to 6.5% SDS-PAGE gels (mini gels, 1.5 mm spacers, 15 wells). The gels were electrobotted onto a sheet of nitrocellulose and P-gp proteins were detected using A52 monoclonal antibody, horseradish peroxidase-conjugated anti-mouse secondary antibody, and enhanced chemiluminescence (Luminata Forte, Millipore Corp., Etobicoke, ON). An equivalent amount of the sample was also loaded onto 10% (v/v) SDS-PAGE gels and subjected to immunoblot analysis with a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control). The signals were imaged and relative levels of immature (150 kDa) and mature (170 kDa) P-gp were determined using Chemidoc™ XRS+ with Image Lab™ software (Bio-Rad).

**Disulfide Cross-linking Analysis**—Cys-less P-gp mutants containing pairs of cross-linkable cysteines in the TM segments (A80C(TM1)/R741C(TM7), I299C(TM5)/F770C(TM8), and T333C(TM6)/L975C(TM12)), intracellular loops (ICLs) (L175C(ICL1)/N820C(ICL3), A259C(ICL2)/W803C(ICL3), and A266C(ICL2)/F1086C(NBD2)), or the NBDs (C431(NBD1)/L1176C(NBD2), L531C(NBD1)/C1074(NBD2), and P517C(NBD1)/L1105C(NBD2)) were transiently expressed in HEK 293 cells at reduced temperature (30 °C) to promote maturation. Cells expressing mutant A80C(TM1)/R741C(TM7) were washed with PBS containing 10 mM dithiothreitol before membrane preparation because the mutant cross-links spontaneously (29). Membranes were prepared and samples were incubated at 0 °C (A80C/R741C, A259C/W803C, I299C/F770C, and A266C/F1086C) or at 20 °C (L175C/N820C, C431/L1176C, and L521C/L1105C) for 10 min in the presence or absence of 0.5
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mm copper phenanthroline (oxidant to promote disulfide bond formation) in the presence or absence of 0.25 μM (for TM segment cysteine mutants) or 1 μM tariquidar (for the ICL and NBD cysteine mutants). Mutant T333C/L975C was cross-linked in whole cells at 20 °C with 0.5 mM BMOE (bismaleimidoethane) cross-linker (Thermo Fisher Scientific, Burlington, ON) in the absence or presence of 0.25 μM tariquidar (30). Mutant P517C/I1050C was cross-linked in membranes at 0 °C with 50 μM 1,4-butanediyldimethanesulfonate (BMTS) in the absence or presence of 1 μM tariquidar (30). The reactions were stopped by addition of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 25 mM EDTA and no reducing agent. The reaction mixtures were then subjected to SDS-PAGE (6.5% (w/v) polyacrylamide gels and 7% gels for membranes at 0 °C with 50 mM tariquidar (30). Mutant P517C/I1050C was cross-linked in membranes at 0 °C with 50 mM tariquidar. The cells were suspended in Tris-buffered saline, pH 7.4 (5 mg/ml of protein). Samples of the membranes were treated for 5 min at 20 °C with various concentrations of TPCP-tarquidar (12,000 BAEE units/mg; Sigma). The reactions were stopped by addition of lima bean trypsin inhibitor (Worthington Biochemical Corp.) followed by 1 volume of 2× SDS buffer. The samples were run on 10% SDS-PAGE gels and subjected to immunoblot analysis.

Data Analysis—The signals from the immunoblots were visualized and quantified using the ChemiDoc™ XRS+ with Image Lab™ software (Bio-Rad). The results were expressed as an average of triplicate experiments ± S.D. The Student’s two-tailed t test was used to determine statistical significance (p < 0.001).

Results

Tariquidar Inhibits Cross-linking between Cysteine Located in the TM Segments—There is no high-resolution structure of human P-gp. A homology model based on the crystal structure of mouse P-gp (16) is shown in Fig. 1A. The 1280 amino acids are organized into two nucleotide-binding domains (NBDs) and two TM and TMD. Each TMD contains 6 TM segments that extend into the cytoplasm to form ICLs that connect the NBDs to the TMDs. The TMDs interlink (TMs 1, 2, 3, 6, 10, and 11 on one side and TMs 7, 8, 9, 12, 4, and 5 on the other side) to form two divergent wings that attach to NBD1 or NBD2. The protein alternates between open and closed conformations during the predicted catalytic cycle (9). P-gp is shown in an open conformation with the NBDs apart and the drug-binding chamber (pocket surrounded by the TM segments) closed to the outside. In the closed conformation, the NBDs come close together in a head-to-tail orientation to form a pair of ATP-binding sites at their interface and the drug-binding pocket is open to the outside.

McCormick et al. (9) predicted that tariquidar could bind at a site composed of ICL2 and ICL3 (site 1) or to one of two sites (sites 2 and 3) located within the TM segments of the TMDs (Fig. 1B). Sites 1 and 2 are in one wing (attached to NBD2), whereas site 3 is in the lipid layer between the TM segments of both wings. One approach that could be used to identify domain interfaces affected by substrate binding is cysteine cross-linking protection assays. Cross-linking of cysteines can be readily detected because a disulfide bond between different domains causes the protein to migrate slower on SDS-PAGE gels (34).

The predicted tariquidar-binding sites all lie at interfaces between domains of the two homologous halves of P-gp. Site 1 is at the interface between ICL2 (N-half) and ICL3 (C-half), whereas sites 2 and 3 lie at the interface between TM segments
in TMD1 and TMD2. Sites 1 and 2 are on one structural wing, whereas site 3 is located at the interface between the two wings (Fig. 1B). Therefore binding of tariquidar at the interface would be predicted to block cysteine cross-linking.

Accordingly, a Cys-less P-gp (35) was used to generate mutants that contained double cysteines at the interfaces involving TM segments of TMD1 and TMD2 (A80C(TM1)/R741C(TM7), I299C(TM5)/F770C(TM8), and T333C(TM6)/L975C(TM12)), ICL2/ICL3 (L175C(ICL1)/N820C(ICL3), A259C(ICL2)/W803C(ICL3), and A266C(ICL2/F1086C(NBD2)) or the interface between NBD1 and NBD2 (C431(NBD1)/L1176C(NBD2), L531C(NBD1)/C1074(NBD2), and P517C(NBD1)/I1050C(NBD2)) were included as controls. Tariquidar was not expected to inhibit cross-linking between the NBDs as by conversion of a core-glycosylated to a mature form of the mutant (2).

The cysteine mutants were then expressed in HEK 293 cells. For cross-linking of most mutants, membranes were prepared, incubated with or without tariquidar, and then subjected to immunoblot analysis. The amount of cross-linked P-gp (% X-link) relative to total P-gp was determined. Each value is the mean ± S.D. (n = 3). An asterisk indicates significant difference (p < 0.001) relative to that cross-linked in the absence of tariquidar.
A266C(ICL2/F1086C(NBD2)) (Fig. 2B) or the NBD (C431(NBD1)/L1176C(NBD2), L531C(NBD1)/C1074(NBD2), and P517C(NBD1)/I1050C(NBD2)) mutants (Fig. 2C). These results suggest that tariquidar likely binds to a site at the interface between TMD1 and TMD2 within the lipid bilayer (sites 2 or 3) rather than at the ICL2/ICL3 interface (site 1). This is supported by the recent observation that 500 nM tariquidar efficiently rescued 25 mutants with processing mutations in the ICL2/ICL3 interface (14 mutants in ICL2 and 11 mutants in ICL3) (34).

Identification of Arginine Mutations That Inhibit Tariquidar Rescue—Tariquidar acts as a potent corrector to rescue P-gp processing mutants (2). P-gp contains three N-glycosylated sites in the extracellular loop that connects TM segments 1 and 2 (Fig. 1A). Wild-type P-gp is initially synthesized as a 150-kDa core-glycosylated protein in the endoplasmic reticulum (ER). The protein then leaves the ER and the carbohydrate is modified in the Golgi to yield a 170-kDa mature protein that is then delivered to the cell surface (maturation). The presence of a processing mutation traps P-gp in the ER as a partially folded protease-sensitive core-glycosylated intermediate that is rapidly degraded. Expression of P-gp processing mutants in the presence of drug substrates promotes maturation of the protein to yield functional P-gp at the cell surface (5). Tariquidar acts as a more potent corrector of P-gp processing mutants as concentrations of only about 100 nM are required for rescue (2) compared with concentrations of at least 2000 nM for P-gp substrates such as cyclosporine A, vinblastine, verapamil, and capsaicin (5).

Arginine mutagenesis has been a particularly useful approach to probe the drug-binding pocket structure of P-gp, orientation of the TM segments, and interactions with drug substrates (43, 44). The rationale was that one face of the TM segments of P-gp surround an aqueous chamber (45), whereas the other face is in contact with the surrounding lipid. Insertion of a highly charged arginine side chain (pKₐ about 12.5) into a lipid face destabilizes a TM segment because of the large free energy barrier of about 17 kcal/mol for exposing it to lipid hydrocarbon (46). Therefore, insertion of an arginine side chain into the aqueous face of a TM segment would not cause destabilization. Destabilization of a P-gp TM segment is readily detectable because it inhibits P-gp maturation. Arginine mutagenesis enabled us to map the orientation of the 12 TM segments (44) and identify the correct structural model of P-gp (14).

P-gp substrates are hydrophobic. Insertion of a highly charged arginine side chain into a binding site of a P-gp processing mutant would inhibit the ability of a drug substrate to promote maturation. We have used the G251V processing mutant for arginine mutagenesis and drug rescue assays because the G251V mutation is outside of the drug-binding pocket (located in ICL2) and it yields the 150-kDa immature form of P-gp as the major product when expressed in the absence of drug substrates (44). Drug substrates promote maturation of G251V to yield 170-kDa mature P-gp as the major product. Rescue of G251V could be blocked by the presence of an arginine side chain. For example, we showed that an M69R mutation specifically blocked rescue of a P-gp G251V processing mutant with verapamil but not rescue with cyclosporine A, vinblastine, or rhodamine B (43). Accordingly, we screened the rescue of 223 G251V processing mutants in which each position in the TM segment was replaced with arginine. HEK 293 cells were transfected with each mutant and expressed for 16 h in the absence or presence of 0.5 μM tariquidar. This concentration of tariquidar was chosen because there was nearly complete rescue of the G251V parent (Fig. 3). If an arginine mutant was not rescued with tariquidar, it was tested for rescue with cyclosporine A to check if the arginine specifically inhibited rescue with tariquidar. Cyclosporine A was found to act as a more potent corrector compared with other drug substrates (5).

Two examples of arginine mutations that inhibited rescue of G251V are shown in Fig. 3. The I868R(TM10) mutation inhibited maturation as 10-fold higher levels of tariquidar (5 μM) were required to promote maturation of the 150-kDa protein to yield mature P-gp as the major product. Rescue with cyclosporine A, however, was similar to the G251V parent. By contrast, the Y307R mutation inhibited rescue with both tariquidar and cyclosporine A. The effects of arginines introduced into the TM segments of TMD1 on rescue of G251V with 0.5 μM tariquidar are shown in

**FIGURE 3.** Arginine residues inhibit rescue of a G251V processing mutant with tariquidar and cyclosporine A. A, whole cell SDS extracts of cells expressing P-gp processing mutant G251V, G251V/I868R, or G251V/Y307R in the presence of various concentrations of tariquidar or cyclosporine A were subjected to immunoblot analysis. The positions of mature and immature P-gps are indicated. B, the level of mature P-gp relative to total was determined. Each value is the mean ± S.D. (n = 3–5). An asterisk indicates significant difference (p < 0.001) relative to samples incubated in the absence of drugs.
Fig. 4. Our goal was to identify arginines that protrude into the drug-binding pocket and could inhibit tariquidar binding and therefore rescue of the mutant. A potential problem is that arginines may inhibit tariquidar rescue since they disrupt protein folding and rescue because they are located at a lipid or TM segment interface. Therefore, we focused on residues predicted to line the drug-binding pocket (Fig. 5) and identify those that inhibit rescue with tariquidar.

In the N-terminal TMD1 domain, the largest number of arginine mutations predicted to line the drug-binding pocket that inhibited tariquidar rescue were located in TM1 (H61R, G64R, L65R, M68R, M69R, and F72R) and TM5 (F303R, I306R, Y307R, S309R, and Y310R) (Fig. 4, A and E). One arginine mutation predicted to line the drug-binding pocket inhibited rescue in TM2 (A129R) (Fig. 4B) and two arginines predicted to line the drug-binding pocket in TM6 (F336R and F343R) were

FIGURE 4. Rescue of G251V arginine mutants in TMs 1–6 with tariquidar. Whole cell SDS extracts of cells expressing the G251V mutants containing arginines at various positions in predicted TM1 (A), TM2 (B), TM3 (C), TM4 (D), TM5 (E), or TM6 (F) in the presence of 0.5 μM tariquidar or the G251V parent in the absence (−) or presence (+) of tariquidar were subjected to immunoblot analysis. The amount of mature P-gp (170-kDa protein) relative to total (mature 170-kDa plus immature 150-kDa protein) (Percent Mature) was quantified. Each value is the mean ± S.D. (n = 3–5). The black dots identify residues predicted to line the drug-binding pocket shown in Fig. 5. An asterisk indicates a significant decrease (p < 0.001) relative to the G251V parent grown in the presence of tariquidar. The insets show the positions of the residues in the TM segments when arranged as α-helical wheels. Arginine mutations predicted to line the drug-binding pocket and show significant difference relative to the G251V parent are shown as black-filled circles.
found to inhibit tariquidar rescue (Fig. 4F). No arginines predicted to line the drug-binding pocket were found to block tariquidar rescue in TM segments 3 or 4 (Fig. 4, C and D).

Tariquidar rescue of the G251V/arginine mutants in TMD2 are shown in Fig. 6. In TMD2, many arginine mutants predicted to line the drug-binding pocket and inhibited tariquidar rescue were identified. These were in TM7 (Q725R, F728R, and F732R) (Fig. 6A), TM10 (V865R, I868R, and G872R) (Fig. 6D), TM11 (F942R, T945R, Q946R, M949R, Y950R, and Y953R) (Fig. 6E), and TM12 (L975R, F978R, and V982R) (Fig. 6F). No arginines predicted to line the drug-binding pocket were found to block tariquidar rescue in TM segments 8 or 9 (Fig. 6, B and C).

A total of 30 arginine mutants predicted to line the drug-binding pocket inhibited rescue of G251V with tariquidar (Figs. 4 and 6). Fig. 3 showed that the Y307R(TM5) mutation but not I868R(TM10) also inhibited rescue with cyclosporine A. Therefore, do other arginine mutations inhibit rescue of G251V with cyclosporine A? To address this question, we tested for rescue of the other 28 arginine mutants with 5 μM tariquidar (Fig. 8). It was found that the G251V/I868R mutant as expression in the presence of 5 μM cyclosporine A yielded mature P-gp as the major product in TM1 (H61R, G64R, L65R, M68R, and M69R), TM5 (F303R, I306R, and S309R), TM7 (Q725R and F728R), TM10 (I868R and G872R), TM11 (F942R, T945R, and Q946R), and TM12 (V982R) (Fig. 7). The other 12 mutants in TM1 (F72R), TM5 (Y307R and Y310R), TM6 (F336R and F343R), TM7 (F728R), TM10 (V865R), TM11 (M949R, Y950R, and Y953R), and TM12 (L975R and F978R) were not rescued by cyclosporine A (Fig. 7). The results suggest that there may be partial overlap of the tariquidar and cyclosporine A binding sites as predicted by Chufan et al. (8).

Arginine Mutations Inhibit Tariquidar-stimulated ATPase Activity—A potential disadvantage of the tariquidar-rescue assay is that it involves rescue of a P-gp processing mutant. Would the arginine mutations disrupt tariquidar interactions in a wild-type background? To address this question, we assayed for tariquidar-stimulated ATPase activity of purified arginine mutants. Drugs modulate P-gp ATPase activity (47) and the level of activation or reduction in basal ATPase activity is highly dependent on the composition of the lipid (48). Tariquidar can act as a stimulator or inhibitor of human P-gp ATPase activity depending on its lipid environment. For example, we and others (8) found that tariquidar and some drug substrates inhibited human P-gp ATPase activity when it is expressed in insect S9 cells as P-gp shows a high basal ATPase activity when incorporated into S9 lipids. P-gp shows a very low basal ATPase activity when incorporated into sheep brain lipid (49) and its ATPase activity is activated by tariquidar (50).

Accordingly, each of the 30 arginine residues (Figs. 4 and 6) was introduced into a histidine-tagged wild-type background. The histidine-tagged mutants were expressed in HEK 293 cells and P-gp isolated by nickel-chelate chromatography (26). The mutants were expressed at 30 °C in the presence of 10 μM cyclosporine A to promote maturation. All of the arginine mutants in the wild-type background yielded mature P-gp as the major product when expressed at low temperature (30 °C) in the presence of cyclosporine A (data not shown). P-gp processing mutants resemble processing mutants of its CFTR sister protein as expression at low temperature (30 °C) promotes maturation of the mutants (50).

The isolated mutant P-gps were mixed with lipid and assayed for ATPase activity in the presence of various concentrations of tariquidar. Wild-type P-gp exhibited basal ATPase activity of about 0.1 μM P/min/mg of P-gp. The ATPase activity was then measured in the presence of various concentrations of tariquidar (0–60 μM). Wild-type P-gp ATPase activity was maximally stimulated about 8-fold (Fig. 8A) and had a half-maximal stimulation (S50) at a concentration of about 0.8 μM tariquidar (Fig. 8B). Seventeen of the 30 G251V/arginine mutants (M68R, M69R, and F72R in TM1; I306R, Y307R, S309R, and Y310R in TM5; F336R in TM6; F728R and F732R in TM7; I868R and G872R in TM10; F942R, T945R, M949R, and S952R in TM11; and V982R in TM12) that could not be rescued with tariquidar showed little or no stimulation of ATPase activity with tariquidar (Fig. 8A). Mutants L65R(TM1), A129R(TM2), F723R(TM7), Y950R(TM11), Y953R(TM11), and F978R(TM12) showed partial activity (about 25–50% of wild-type activity), whereas the maximal tariquidar-stimulated ATPase activity of mutants H61R(TM1), G64R(TM1), F303R(TM5), F343R(TM6), Q725R(TM7), V865R(TM10), Q946R(TM11), and L975R(TM12) was about 50–100% of wild-type enzyme. All of the active arginine mutants, however, had S50 concentrations that were at least 5-fold higher than wild-type P-gp (Fig. 8B).

The decrease in the affinities of these mutants for tariquidar in their ATPase activities was consistent with their inability to be rescued with tariquidar (Figs. 4 and 6). These results indicate that the arginine side chain in these 30 mutants perturbs P-gp/tariquidar interactions. The locations of these residues are shown in Fig. 9. They overlap with many residues predicted in silico to be present in site 3 (9) of the tariquidar-binding sites (Fig. 1B).

3 T. W. Loo and D. M. Clarke, unpublished data.
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Tariquidar Promotes Maturation of TMD1 into a Protease-resistant Conformation—Rescue of processing mutants by tariquidar and cyclosporine A showed differences because many mutants containing processing mutations outside the drug-binding pocket could only be rescued with tariquidar (2). Tariquidar was about 10 times more potent than cyclosporine A in rescuing most processing mutants. This suggested that both compounds might rescue processing mutants by different mechanisms.

One possibility is that rescue by tariquidar resembles the rescue of CFTR processing mutants with VX-809 (22). Corrector VX-809 is the most effective pharmacological chaperone to repair CFTR processing mutants (51). Its mechanism of rescue involves stabilization of TMD1 to yield a protease-resistant protein (22). By contrast, the mechanism of cyclosporine A rescue of P-gp mutants involves stabilization of TMD2 (27). Cyclosporine A did not promote maturation

FIGURE 6. Rescue of G251V arginine mutants in TMs 7–12 with tariquidar. Whole cell SDS extracts of cells expressing the G251V mutants containing arginines at various positions in predicted TM7 (A), TM8 (B), TM9 (C), TM10 (D), TM11 (E), or TM12 (F) in the presence of 0.5 μM tariquidar or the G251V parent in the absence (−) or presence (+) of tariquidar were subjected to immunoblot analysis. The amount of mature P-gp (170-kDa protein) relative to total (mature 170-kDa plus immature 150-kDa protein) (Percent Mature) was quantified. Each value is the mean ± S.D. (n = 3–5). The black dots identify residues predicted to line the drug-binding pocket shown in Fig. 5. An asterisk indicates a significant decrease (p < 0.001) relative to the G251V parent grown with tariquidar. The positions of the residues in the TM segments when arranged as α-helical wheels are shown in the insets. The black-filled circles represent arginine mutations predicted to line the drug-binding pocket and show significant difference relative to the G251V parent.
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FIGURE 7. Rescue of G251V arginine mutants with cyclosporine A. Cells expressing the G251V parent or G251V/arginine mutants that could not be rescued with tariquidar (identified as black circles in the α-helical wheels shown in Figs. 4 and 6) were expressed in the presence of 5 μM cyclosporine A. Whole cell SDS extracts were subjected to immunoblot analysis. The amount of mature P-gp (170-kD protein) relative to total (mature 170-kD protein) (Percent Mature) was quantified. Each value is the mean ± S.D. (n = 3–5). An asterisk indicates a significant difference (p < 0.001) relative to the G251V parent grown in the presence of cyclosporine A.

FIGURE 8. Mutants defective in tariquidar rescue show reduced tariquidar-stimulated ATPase activity. Histidine-tagged wild-type or mutant P-gps (in wild-type background) containing arginines defective in tariquidar rescue (identified as black circles in the α-helical wheels shown in Figs. 4 and 6) were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated P-gps were mixed with lipid and ATPase activities were measured in the presence of a saturating concentration of tariquidar to determine the concentration required to stimulate ATPase activity by 50% (S50) or in the presence of various concentrations of tariquidar to determine maximal activity (Amax). Activity by 50% (S50) (B) were expressed in the presence of 5 μM cyclosporine A. The isolated P-gps were mixed with lipid and ATPase activities were measured in the presence of 5 μM cyclosporine A. Whole cell SDS extracts were subjected to immunoblot analysis (Fig. 10). Maturation of TMD1 in the presence of tariquidar occurred only when TMD1 was co-expressed with TMD2 (Fig. 10, A and B).

A52-tagged TMD1 together with untagged TMD2 were co-expressed in HEK 293 cells in the presence of tariquidar. The cells were solubilized and treated with endoglycosidases H (Hf) and F (PNGase F). Samples were then subjected to immunoblot analysis. Fig. 10C shows that tariquidar promoted maturation of TMD1 such that the mature product was resistant to endoglycosidase H but not to endoglycosidase F. By contrast, the immature form of TMD1 was sensitive to both endoglycosidases. These results indicate that the immature product is in the ER, whereas the mature form of TMD1 has traversed the Golgi apparatus.

We previously showed that P-gp folds into a more compact conformation when it leaves the ER such that it becomes more resistant to protease digestion (27). Therefore, we tested whether the mature form of TMD1 also became resistant to protease digestion. Membranes were prepared from HEK 293 cells co-expressing A52-tagged TMD1 and untagged TMD2 and expressed in the absence or presence of tariquidar. Samples of the membranes were then treated with various concentrations of TPCK-trypsin for 5 min at 20 °C. It was found that the mature form of TMD1 was over 100-fold more resistant to trypsin digestion than the immature form (Fig. 10, D and E). About 10 μg/ml of trypsin was required to reduce the amount of immature TMD1 by 50%, whereas mature TMD1 remained resistant to trypsin at the highest concentration tested (1000 μg/ml) (Fig. 10E).

The results showed that tariquidar differed from cyclosporine A. We previously reported that cyclosporine A did not stabilize TMD1 (27). Here we showed that tariquidar promoted maturation of the TMD1 protein into a protease-resistant conformation.
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**FIGURE 10. Tariquidar stabilizes TMD1.** A, A52-tagged TMD1 (T1) (residues 1–379), A52-tagged TMD2 (T2) (residues 681–1025), or both A52-tagged TMD1 plus A52-tagged TMD2 (T1 + T2) were expressed in the absence (−) or presence (+) of 1 μM tariquidar (Tar). Whole cell SDS extracts were subjected to immunoblot analysis. The locations of TMD2 (T2) and mature and immature forms of TMD1 (T1) relative to total (mature and immature TMD1) (% Mature T1) was quantified. Each value is the mean ± S.D. (n = 3–5). An asterisk indicates a significant difference (p < 0.001) relative to samples grown in the absence of tariquidar. C, HEK 293 cells expressing A52-tagged TMD1 plus untagged TMD2 and grown in the presence of tariquidar were treated without (−) or with (+) endoglycosidase H (Endo H) or PNGase F. The reactions were stopped by addition of SDS sample buffer and subjected to immunoblot analysis. The positions of mature, immature, and unglycosylated (Unglycos) forms of TMD1 are indicated. D, membranes prepared from cells expressing A52-tagged TMD1 plus untagged TMD2 and grown in the presence of tariquidar were treated without (−) or with (+) trypsin. The reactions were stopped by addition of trypsin inhibitor and samples were subjected to immunoblot analysis. The positions of mature and immature forms of TMD1 are indicated. E, the levels of mature (filled bars) or immature (unfilled bars) forms of TMD1 were determined and the amount remaining (% Remaining) after treatment with various levels of trypsin was compared with the untreated control. An asterisk indicates a significant difference (p < 0.001; n = 3) relative to that without trypsin.

**Discussion**

Through arginine mutagenesis we identified 30 positions (Fig. 11A) in the predicted drug-binding pocket where arginines caused major disruptions in the ability of tariquidar to rescue processing mutants or activate ATPase activity. McCormick et al. (9) used molecular docking studies to identify two potential tariquidar-binding sites in the predicted drug-binding domain (sites 2 and 3). When we compared the results of the arginine mutagenesis studies to the molecular docking predictions, it was observed that 17 of the 20 residues predicted to lie close to the tariquidar-binding site in site 3 showed overlap (Fig. 11B). By contrast, only 2 of 13 residues of the predicted site 2 tariquidar-binding site showed overlap with residues identified by arginine mutagenesis (Fig. 11C). Therefore, the results of the cross-linking, arginine mutagenesis studies, and molecular docking predictions suggest that predicted site 3 is the likely high affinity-binding site for tariquidar.

Binding of tariquidar to site 3 might help explain the mechanism of its effectiveness as a P-gp corrector and inhibitor. Site 3 is located in the lipid bilayer at the interface between the two wings of P-gp (Fig. LB). Binding of tariquidar at this site would enable it to stabilize both TMDs (enhanced corrector activity) and bring the two wings and NBDs together into a closed conformation that would result in stimulation of ATPase activity but with no efflux activity (inhibitor). This would be consistent with the observation that tariquidar appears to trap P-gp in a closed conformation (2).

We identified 13 additional arginine mutations (H61R, G64R, L65R, and M68R in TM1; A129R in TM2; I306R and S309R in TM5; F343R in TM6; F942R, T945R, Q946R, and Y950R in TM11; and L975R in TM12) (Fig. 11A) that were not predicted to lie within 4.5-Å of the predicted site 3 tariquidar-binding site (9). Chufan et al. (8) also identified three other different residues (Leu65(TM1), Gln946(TM11), and Leu975(TM12)) that were in agreement with our results. Because there is no high-resolution structure of human P-gp, the different residues identified to be involved in tariquidar binding by the two in silico methods could be due to different algorithms and programs used to generate the homology models of human P-gp for the docking studies. It could also be due to the scoring function chosen for best docking (52). The lowest binding energy score is often chosen in docking studies, but this might not be the correct pose that reflects actual drug binding (52).
A reason for the identification of additional residues in our study that were not predicted by the in silico docking studies was that some arginines might have perturbed the structure of TM segments. For example, 8 of the 13 arginines with reduced tariquidar interactions are located in TM1 and TM11 (Fig. 11A). Cysteine cross-linking studies showed that TM segments 1 and 11 in human P-gp were adjacent to each other and that interactions between TM segments 1 and 11 were altered during the catalytic cycle by ATP hydrolysis (53). In addition, suppressor mutations in TM1 were found to increase folding and maturation of P-gp processing mutations by promoting interactions between TM segments 1 and 11 (43). Therefore, arginines predicted to lie outside of the tariquidar-binding site in TM1 and TM11 in the docking studies (H61R, G64R, L65R, and M68 in TM1; F942R, T945R, Q946R, and Y950R in TM11) might alter interactions between TM1 and TM11.

Finally, another explanation for differences between the arginine mutagenesis and in silico docking studies (8, 9) is that there is evidence that P-gp recognizes drug substrates by an induced-fit mechanism (54). For example, binding of drug substrates was found to change the cross-linking pattern between TM segments 6/11 and 6/12 (54). Changes in the cross-linking pattern suggested that binding of a drug could induce repacking or rotation of the TM segments (54).

This study shows that in silico docking studies are potentially valuable tools in aiding the mapping of binding site(s) of a drug substrate/inhibitor by biochemical methods. The results in this study suggest that tariquidar binds to a binding site that is within the TMDs and is consistent with the idea that there is a large flexible drug-binding pocket with the TMDs that can accommodate other drug substrates (16, 55–58). It is unknown why tariquidar is not transported like other drug substrates even though it still stimulates ATPase activity. It is possible that tariquidar is not transported because it binds with a very high affinity for P-gp (59) to activate ATPase activity but is not released during the catalytic cycle (4) or it could simply move from a high-affinity site to a low-affinity site or to a silent non-transported site and bound in a different orientation within the drug-binding pocket during the catalytic cycle. This could simply involve a change in the orientation of tariquidar in the drug-binding pocket. This behavior is not unusual as labeling studies of P-gp cysteine mutants with thiol-reactive substrates of verapamil (60, 61) or rhodamine B (55) could covalently attach to different residues within the drug-binding pocket and still activate ATPase activity (31, 60–62). Therefore it appears that drug substrates could bind in different orientations within the drug-binding pocket.

Tariquidar is a transport substrate of BCRP, another ABC drug pump. BCRP functions as a dimer with each monomer consisting of one NBD and a TMD made up of 6 TM segments (63). BCRP is different from P-gp because it has a much lower affinity for tariquidar (4). In ATPase assays performed in membrane preparations overexpressing the proteins, a 50% change in ATPase activity was observed with about 5 nM tariquidar for P-gp (59). BCRP required about 30-fold higher levels (about 150 nM) of tariquidar to activate ATPase activity by 50% (4). It should be noted that we required about 100-fold higher levels of tariquidar in the ATPase assays (about 500–1000 nM) because the presence of detergent increases the apparent affinity of drug substrates for P-gp by 100-fold or more (64).

We found that the mechanism of rescue with tariquidar was different from that with cyclosporine A because it could promote maturation and stabilize TMD1 (Fig. 10). Conversion from a protease-sensitive conformation to a protease-resistant conformation appears to be an important step in P-gp maturation (65). Processing mutations trap the protein in the ER in a loosely folded conformation with incomplete packing of the TM segments. Drug substrates promote packing of the TM segments to induce the protein to complete the folding process.

CFTR shows a similar mechanism. CF mutations like AF508 inhibit maturation by trapping the protein in a protease-resistant pre-folded state (66) with incomplete packing of the TM segments (67). Specific correctors can bind to the protein (68) in the ER (69) to promote maturation. Unfortunately, the correctors identified to date only promote maturation of a small fraction of the molecules, and hence are poor therapeutic candidates. Efficient correctors also need to be developed for other ABC processing mutants that cause disease such as ABCG2 (gout), ABCB4/ABCB11 (progressive familial intrahepatic cholestasis), ABCA1 (Tangier disease), ABC2 (Dubin-Johnson syndrome), ABCG6 (pseudoxanthoma elasticum), and ABCB8 (hyperinsulinimic hypoglycemia of infancy).

Identification of a universal target to repair ABC processing mutants would aid in the development of correctors for different diseases. Our studies on CFTR and P-gp suggest that TMD1 would be an attractive target because the best correctors for P-gp (tariquidar) and CFTR (VX-809) stabilized the TMD1 domain. Our tariquidar mapping studies suggest that molecular docking studies can be a useful approach to identify a target site.

Author Contributions—Both authors designed and conducted the experiments. Both authors analyzed the data, wrote the article, and approve of the version to be published.

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