Published in final edited form as:

Biochem Pharmacol. 2016 February 1; 101: 40–53. doi:10.1016/j.bcp.2015.12.007.

Drug-Protein Hydrogen Bonds Govern the Inhibition of the ATP Hydrolysis of the Multidrug Transporter P-glycoprotein

Eduardo E. Chufan, Khyati Kapoor, and Suresh V. Ambudkar*

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4256, USA

Abstract

P-glycoprotein (P-gp) is a member of the ATP-Binding Cassette transporter superfamily. This multidrug transporter utilizes energy from ATP hydrolysis for the efflux of a variety of hydrophobic and amphipathic compounds including anticancer drugs. Most of the substrates and modulators of P-gp stimulate its basal ATPase activity, although some inhibit it. The molecular mechanisms that are in play in either case are unknown. In this report, mutagenesis and molecular modeling studies on P-gp led to the identification of a pair of phenylalanine-tyrosine structural motifs in the transmembrane region that mediate the inhibition of ATP hydrolysis by certain drugs (zosuquidar, elacridar and tariquidar), with high affinity (IC_{50} 's ranging from 10 to 30 nM). Upon mutation of any of these residues, drugs that inhibit the ATPase activity of P-gp switch to stimulation of the activity. Molecular modeling revealed that the phenylalanine residues F978 and F728 interact with tyrosine residues Y953 and Y310, respectively, in an edge-to-face conformation, which orients the tyrosines in such a way that they establish hydrogen-bond contacts with the inhibitor. Biochemical investigations along with transport studies in intact cells showed that the inhibitors bind at a high affinity site to produce inhibition of ATP hydrolysis and transport function. Upon mutation, they bind at lower affinity sites, stimulating ATP hydrolysis and only poorly inhibiting transport. These results also reveal that screening chemical compounds for their ability to inhibit the basal ATP hydrolysis can be a reliable tool to identify modulators with high affinity for P-gp.

Graphical abstract

Corresponding Author: Suresh V. Ambudkar, Ph.D., 37 Convent Drive, Building 37, Room 2120, LCB, CCR, NCI, NIH, Bethesda, MD 20892-4256, Phone: +1 (301) 402 4178, FAX: +1 (301) 435 8188, ambudkar@helix.nih.gov.

Chemical compounds studied in this article

Zosuquidar (PubChem CID: 153997); Elacridar (PubChem CID: 119373); Tariquidar (PubChem CID: 148201); Cyclosporine A (PubChem CID: 5284373); Verapamil (PubChem CID: 62969); Nilotinib (PubChem CID: 644241); Valinomycin (PubChem CID: 5649); Calcein-AM (PubChem CID: 4126474); Rhodamine 123 (PubChem CID: 65217); Daunorubicin (PubChem CID: 30323).

Conflicts of interest

The authors declare no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

ABC transporter; drug-binding site; multidrug resistance; modulators; structural motifs

1. Introduction

P-glycoprotein (P-gp, ABCB1) is a member of the ATP-Binding Cassette (ABC) transporter superfamily. It is a single, glycosylated polypeptide of about 170 kDa, expressed at the apical surface of epithelial cells in the intestines, kidney, liver, adrenal gland, blood-brain barrier, and placenta. This transporter utilizes energy from ATP hydrolysis for the efflux of a variety of hydrophobic and amphipathic compounds including anticancer drugs [1, 2]. It plays an important role in the pharmacokinetics of many drugs, altering absorption, distribution, metabolism, and excretion. The expression of P-gp at the surface of tumor cells is also a contributing factor in the development of multidrug-resistant cancer. P-gp consists of two homologous halves, each containing six transmembrane helices and a nucleotide-binding domain (NBD). There is consensus among researchers that P-gp alternates between an inward-facing conformation with separated NBDs (inverted V-shape) competent for substrate binding, and an outward-facing conformation with united NBDs (V-shape) consistent with substrate release outside the cell. The X-ray crystal structures of mouse and *C. elegans* P-gp are representative structures of the inward-facing conformation [3–5], although the extent of domain separation in physiological conditions is a matter of debate. The X-ray structure of bacterial SAV1866 with bound ADP is representative of the outward-facing conformation [6]. Using this alternating access mechanism, substrate translocation is powered by ATP hydrolysis. Hence, most substrates and modulators stimulate the basal ATPase activity of P-gp [7]. Interestingly, a few drugs (zosuquidar, elacridar and tariquidar) have been reported to inhibit the basal ATP hydrolysis of P-gp. These drugs also happen to be potent inhibitors of P-gp transport [8–10]. They are third generation modulators of P-gp that inhibit drug transport and ATPase activity at nanomolar concentrations [11]. Further, it has been recently demonstrated that the oral co-administration of paclitaxel and docetaxel (anticancer agents) with elacridar increases plasma levels of the taxanes, thus supporting the therapeutic strategy of co-administration of drugs with a potent inhibitor of P-gp [12].

We found that mutation of polar residues that are capable of establishing hydrogen bond (H-bond) interactions with inhibitors at the drug-binding pocket of P-gp dramatically changes the typical biochemical behavior of P-gp. Drugs that usually inhibit basal ATP hydrolysis switch to stimulation when two tyrosines and one glutamine are mutated (Y307A/Q725A/

Y953A). Two phenylalanine residues (F728 and F978) were also found to be essential to the inhibition profile. Molecular modeling studies revealed that the phenylalanine residues orient the aromatic ring of the tyrosine residues (Y310 and Y953) in a manner such that effective H-bond interactions are established between the protein and the drugs. Transport data showed the inhibition of the P-gp function by these drugs depends on their ability to inhibit ATP hydrolysis. When drugs lose the ability to inhibit ATP hydrolysis, they also lose the ability to reverse transport with high affinity [IC_{50} (cysless WT) = 5–10 nM while IC_{50} (Y307A/Q725A/Y953A) > 200 nM]. Based on these results, we propose that screening compounds for their ability to inhibit basal ATP hydrolysis with high affinity is a reliable method to identify high affinity modulators of P-gp and possibly of other ABC drug transporters.

2. Materials and methods

2.1. Chemicals

The chemical compounds under investigation, zosuquidar, tariquidar and elacridar were purchased from Selleck Chemicals (Houston, TX), MedKoo Biosciences (Chapel Hill, NC), and Sigma-Aldrich Chemical Co. (St. Louis, MO), respectively. Cyclosporine A was obtained from Alexis Corporation (Lausen, Switzerland). The radioactive compound [^{125}I]iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). The fluorescent compounds calcein-AM, bodipy-FLprazosin and bodipy-paclitaxel were purchased from Invitrogen (Carlsbad, CA). NBD-cyclosporine A was generously provided by Drs. Anika Hartz and Björn Bauer, University of Minnesota (Duluth, MN). ATP, valinomycin and all other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The P-gp specific monoclonal antibody C219 was supplied by Fujirebio Diagnostic Inc. (Malvern, PA); while the antibodies used for flow cytometry studies MRK16 and UIC2 were purchased from Kyowa Medex Company (Tokyo, Japan) and eBioscience (San Diego, CA), respectively. FITC-labeled anti-mouse secondary antibody IgG2a was obtained from BD Biosciences (San Jose, CA).

2.2. Cell lines and culture conditions

HeLa cells were cultured in DMEM media as described previously [13].

2.3. BacMam baculovirus transduction of HeLa cells

HeLa cells were transduced with the cysless-WT or mutant P-gps (Y307A, Q725A, Y953A and triple mutant) BacMam virus at a titer of 50–60 particles per cell as described previously [14]. The cells were trypsinized after 24 hours, counted and analyzed by flow cytometry for cell surface expression and function.

Cysless-WT and mutant P-gp-expressing cells (250,000 cells) were analyzed for cell surface expression by 60 minutes incubation with MRK16 antibody (1 μ g per 100,000 cells). Cells were subsequently washed and incubated with FITC-labeled anti-mouse secondary antibody IgG2a (1 μ g per 100,000 cells) [14]. The cells were then analyzed by flow cytometry using a FACSort instrument (488 nm argon laser and 530 nm bandpass filter).

2.4. UIC2 Shift assay

The UIC2 shift assay was performed as described earlier [14]. Briefly, 250,000 cells were resuspended in 0.5 ml of IMDM and allowed to equilibrate at 37°C in a water bath for 5 min with 20 µM of P-gp substrate, cyclosporine A, or DMSO as a control. Cells were then incubated with UIC2 antibody (1 µg per 100,000 cells) at 37°C for another 30 min. They were subsequently washed and resuspended in IMDM, and incubated with FITC-labeled anti-mouse secondary antibody (1 µg per 100,000 cells) for another 30 min at 37°C. The fluorescence of FITC was analyzed by flow cytometry as described previously [14]. The UIC2 shift was calculated as the difference between UIC2 binding in the presence and in the absence of cyclosporine A. The UIC2 shift was compared for all mutant P-gps with respect to cysless-WT.

2.5. Transport of fluorescent substrates

The transport function of cysless-WT and mutant P-gps was determined using flow cytometry as previously explained [14]. Cells were incubated with fluorescent substrates such as calcein-AM (0.5 µM) for 10 min; or JC-1 (0.5 µM), rhodamine123 (1.3 µM), NBDCsA (0.5 µM), daunorubicin (0.5 µM), bodipy-FL-prazosin (0.5 µM), and bodipy-vinblastine (0.5 µM) for 45 min. They were subsequently washed with cold PBS and resuspended in PBS containing 0.1% BSA before analysis. The accumulation of substrates was measured with respect to the accumulation in non-functional P-gp mutant E556Q/E1201Q [15], which was taken as a negative control for all transport experiments.

2.6. Reversal of NBDCsA or Rh123 transport by tariquidar and zosuquidar by HeLa cells expressing cysless WT and P-gp mutants

HeLa cells (250,000) transduced with either cysless WT or mutant P-gps were treated with varying concentrations (0–2.5 µM) of tariquidar and zosuquidar. The fluorescent substrates NBDCsA (0.5 µM) or Rh123 (1.3 µM) were then added and the cells suspensions were incubated at 37°C for 45 minutes. Cells were washed with cold PBS and resuspended in PBS containing 0.1% BSA before flow cytometry analysis using the above described FACSsort instrument. The software GraphPad Prism 6.0 was used for data analysis.

2.7. Preparation of crude membranes from High-Five insect cells

Crude membranes were prepared by hypotonic lysis and differential centrifugation of P-gp expressing High-Five insect cells [17, 18]. Cells (Invitrogen, Carlsbad, CA) were previously infected with recombinant baculovirus carrying the human *MDR1* cDNA cysless WT (in which all seven cysteine residues are replaced with alanine) and I306C, Y307A, Y307C, Y310A, F335C, F343C, Q725A, Q725C, F728C, M949C, Y953A, Y953C, Y953F, L975C, F978C, V982C, triple mutant Y307A/Q725A/Y953A, and quadruple mutant Y307C/Q725C/V982C/Y953A P-gp (all these mutants in a cysless background), with a His₆-tag at the C-terminus as described previously [16].

2.8. Photo-Crosslinking with [¹²⁵I]-Iodoarylazidoprazosin (IAAP)

Crude membranes of P-gp expressing High-Five insect cells in 50 mM MES-Tris buffer (pH 6.8) were incubated for 10 minutes at 37°C in the absence (control) and presence of

compound(s). The concentration of protein per tube was 60–80 µg per 100 µL volume. Then the samples were transferred to a 4°C bath under subdued light for the addition of 4–7 nM of IAAP. Samples were photo-crosslinked under the effect of UV light for 5 minutes, using a 365 nm UV-lamp. Finally, protein bands were separated by electrophoresis and quantification of IAAP incorporation into P-gp bands was carried out as explained previously [18].

2.9. ATP hydrolysis measurements

ATP hydrolysis was measured in crude membranes prepared from High-Five insect cells expressing cysless WT or mutant P-gp, containing 10 to 25 µg protein per 100 µL. Membranes were incubated in the presence and absence of sodium orthovanadate (0.3 mM), in a 50 mM MES-Tris pH 6.8 buffer containing 50 mM KCl, 10 mM MgCl₂, 5 mM NaN₃, 1 mM EGTA, 1 mM ouabain, and 2 mM DTT. Basal ATPase activity was measured in the absence of any drug and drug-modulated activity was measured in the presence of drugs added as a small aliquot of 100X stock in dimethyl sulfoxide (final concentration of DMSO was kept at 1%). The reaction was started by addition of 5 mM ATP and continued for 20 minutes at 37°C. The reaction was stopped by addition of 5% SDS and the amount of inorganic phosphate generated was quantified with a colorimetric reaction [19].

2.10. Molecular modeling studies

Docking studies of the drugs under investigation were carried out using a homology model of human P-gp based on the recently improved X-ray structures of mouse P-gp [3]. The homology model was provided by Dr. Tanaji Talele and built on the basis of the QZ59-SSS bound X-ray structure of mouse P-gp (4M2T.pdb) [20]. The model is in an *open* inward-facing apo conformation, which is competent for drug binding. The molecular structure of the ligands were prepared as previously described [21]. The docking studies were performed using AutoDock Vina [22] and the receptor and ligand structures were prepared with MGL tools software package [23]. Flexible receptor docking was executed for all the jobs. The side-chains of the residues that interact with the inhibitors in the X-ray structures of mouse P-gp in complex with QZ59-RRR and QZ59-SSS, were selected to use as flexible: L65, M68, M69, F72, Q195, F303, I306, Y307, F336, L339, I340, F343, Q347, N721, Q725, F728, F732, M949, Y953, F957, L975, F978, F983, V982, M986, and Q990.

A receptor grid centered at the position of the bound inhibitor QZ59-RRR ($x=19.317$, $y=52.588$ and $z=-0.676$) and an inner box of dimensions of $40\text{\AA} \times 35\text{\AA} \times 35\text{\AA}$ were used to search for binding poses for the ligands. The exhaustiveness level was set to 40 for all jobs, which is 5 times higher than the default value (8), to reduce the probability of not finding the global minimum of the scoring function, considering the relatively large search box and the elevated number of flexible residues.

3. Results

3.1. Inhibition switches to stimulation of basal ATPase activity of P-gp when polar residues are mutated

We previously showed that residues Y307 and Q725 form part of the primary drug-binding site of human P-gp for several drugs [21]. Cakil et al. showed that Y953 is part of the drug-binding site for propafenones and rhodamine 123, although this tyrosine seems to not be part of the preferential site for rhodamine 123. They also showed that Y953 interacts with these compounds specifically through hydrogen-bonding [24]. All three residues Y307, Q725 and Y953 have been recently shown to be part of the tariquidar-binding site [25]. These polar residues were then individually mutated to alanine in single mutants, and all three were mutated to generate a triple mutant, Y307A/Q725A/Y953A (referred as Triple A P-gp). These mutations were created in a cysless background [26]. Therefore, cysless P-gp is referred to here as cysless wild type (WT) P-gp and represents the control protein. The cysless WT and mutants were expressed in High-Five insect cells for biochemical studies as well as in BacMam baculovirus-transduced HeLa cells for characterization of cell surface expression and transport function. The expression of all mutants studied in both insect and HeLa cells was comparable to cysless WT P-gp (data not shown).

As the level of expression of WT and mutant P-gps is quite high (1–2% of total membrane protein), crude membranes of High-Five insect cells expressing cysless WT and mutant P-gps were used to measure basal and drug-stimulated ATPase activity [21]. Crude membranes have also the advantage the protein transporter is embed in its natural environment, in comparison to purified protein. The K_m for ATP hydrolysis by cysless WT ($K_m = 0.47 \pm 0.03$ mM) and triple A (Y307A/Q725A/Y953A) mutant P-gp ($K_m = 0.66 \pm 0.04$ mM) were essentially the same, thus revealing the affinity for ATP was not affected upon mutation of the three polar residues at the drug-binding pocket of P-gp. Zosuquidar, tariquidar and elacridar inhibited the basal activity to different degrees. Zosuquidar almost completely inhibited ATPase activity, while inhibition by tariquidar and elacridar was approximately 70–80% (Figures 1 and 2). The K_m for ATP hydrolysis by cysless WT slightly decreased in the presence of zosuquidar and elacridar (Figure 1). However, this insignificant decrease cannot explain the inhibition effect. The inhibition must therefore occur when the rate of ADP dissociation is decreased, which is the rate-limiting step in the catalytic cycle of P-gp [17, 27, 28].

Interestingly, when Y307, Q725 and Y953 were mutated to alanine (Y307A/Q725A/Y953A), inhibition switched to stimulation in each case (Figure 2). The stimulation was concentration-dependent, reaching a maximum level at 0.5–1 μ M. Note that the IC_{50} values for inhibition of cysless WT activity was in 10–30 nM range (Figure 2, black curves). On the other hand, drugs that typically stimulate the basal ATP hydrolysis of cysless WT P-gp also stimulated the hydrolysis of ATP by the triple A mutant (Table 1). This particular effect is clearly due mainly to the mutation at Y953, as illustrated in Figure 3, although mutations at Y307 and Q725 are also important for inhibition by tariquidar. The possibility that the switch effect is caused specifically by substitution of a tyrosine residue with alanine was explored by studying the effect of substitution with cysteine and phenylalanine (Y953→C

and F). As shown in Figure 3, the effects observed for Y953→C and Y953→F were essentially the same as found for the Y953→A mutant in the case of zosuquidar, clearly demonstrating that neither substitution can reproduce the physiochemical properties of the original tyrosine. Most importantly, the substitution with phenylalanine (Y953F) demonstrated specifically the contribution of the hydroxyl group to drug binding, constituting strong evidence that Y953 interacts with zosuquidar through H-bonding.

The substitution of Y953 with alanine or cysteine also produced the switch from inhibition to stimulation in the case of elacridar, although mutation to phenylalanine gave an intermediate result ($5 \pm 9\%$ inhibition, at $1 \mu\text{M}$ elacridar) between what was observed for Y953A ($83 \pm 4\%$ stimulation) and cysless WT P-gp ($74 \pm 7\%$ inhibition) (Figure 3 and Table 3). An explanation for this can be found in the fact that elacridar establishes H-bonds not only with Y953, but also with Y310 (see below). Phenylalanine 953 may facilitate elacridar binding at approximately the same position as in WT, thus making possible the formation of an H-bond with Y310. In this case, intermediate results would be expected. With respect to tariquidar, the Y953F mutant exhibited a behavior even closer to WT, $25 \pm 9\%$ inhibition *versus* $63 \pm 8\%$ inhibition observed with cysless WT P-gp ($0.125 \mu\text{M}$ tariquidar) (Figure 3 and Table 4). This behavior is reasonable, considering that tariquidar interacts through H-bonds with several residues (Y310, Y307, Q725) in addition to Y953.

The importance of the mutation at Y953 in the switch effect observed with zosuquidar and elacridar was further corroborated by studies with another triple mutant that lacks the mutation at Y953. The triple mutant is Y307C/Q725C/V982C [21], here called Triple C. Zosuquidar and elacridar inhibited the basal ATPase activity of the Triple C mutant, thus demonstrating that the switch effect is specific for mutation at Y953. When the mutation at Y953 was added to the Triple C mutant, Y307C/Q725C/V982C/Y953A, drugs consequently stimulated its ATPase activity (Tables 2 and 3). This quadruple mutant Y307C/Q725C/V982C/Y953A, which also exhibited the switch effect, was expressed to the same level as WT in insect cells and with a similar level of basal ATPase activity (data not shown).

Is the switch from inhibition to stimulation due specifically to substitutions of polar residues? To answer this question, non-polar residues of the drug-binding pocket were screened for their effect on the modulation of the basal ATP hydrolysis of P-gp upon mutation. Non-polar residues such as valine, leucine, isoleucine, phenylalanine and methionine, which contribute to the primary drug-binding site of P-gp, were mutated to cysteine. The following single mutants were expressed in High-Five insect cells: I306C (TM5), F335C (TM6), F343C (TM6), F728C (TM7), M949C (TM11), L975C (TM12), F978C (TM12) and V982C (TM12) (TM=Transmembrane helix); and modulation of their basal ATPase activity was determined. Mutations of most non-polar residues did not produce the switch effect, although a decrease in inhibitory potency was observed for some (Figure 4). Interestingly, mutation of one phenylalanine (F978, zosuquidar) and two phenylalanine residues (F978C or F728C, elacridar and tariquidar) also produced the switch effect for the drugs given in parentheses. In summary, the inhibitory potency of zosuquidar, elacridar and tariquidar observed for cysless WT P-gp was lost when residues Y953-F978 (zosuquidar), Y953-F978-F728 (elacridar), and Y953-F978-F728-Y307-Q725 (tariquidar) were mutated.

3.2. Apparent binding affinities decrease upon mutation of polar residues

Among inhibitors, zosuquidar, elacridar and tariquidar bind to cysless WT P-gp with high affinity. Apparent binding affinities were determined for these three drugs by measuring their ability to inhibit the basal ATP hydrolysis of P-gp (see Figure 2). The observed IC₅₀ values were in the nanomolar range for the cys-less WT. The apparent affinities significantly decreased when the three polar residues were mutated to alanine (Triple A mutant). These results indicate that drug-protein H-bonds are responsible for critical interactions that mediate the high affinity inhibition of ATP hydrolysis.

3.3. Drugs do not inhibit the photo-crosslinking of the Triple A (Y307A/Q725A/Y953A) mutant with IAAP

IAAP-labeling experiments were carried out to investigate whether or not the drugs under study bind to the same site on the Triple A mutant. The primary binding site of P-gp was previously defined as the site where drugs bind in cysless WT P-gp and inhibit the photo-crosslinking of the transporter with [¹²⁵I]-iodoarylazidoprazosin (IAAP) [21]. Results clearly show that drugs do not bind at the primary site when residues Y307, Q725 and Y953 are mutated to alanine, because they do not inhibit the IAAP-labeling of the Triple A mutant (Figure 5). Instead, they bind at secondary/alternate sites, and produce a stimulatory effect on the ATPase activity of the triple A mutant (see above).

Interestingly, some mutations (such as F343C) have virtually no effect because the drugs inhibit the ATPase activity and the IAAP-photocrosslinking to the same extent as cysless WT P-gp (Figure 6). In contrast, for most of the mutants, the drugs inhibited IAAP-photocrosslinking to a much lower extent than for cysless WT P-gp, consistent with ATP hydrolysis data (Figure 6).

3.4. Triple A (Y307A/Q725A/Y953A) mutant P-gp displays normal cell surface expression

The BacMam baculovirus system was used to express the cysless WT, Triple A and other mutant P-gps in HeLa cells. The cell surface expression and transport function of the mutant was evaluated using flow cytometry, as described in Materials and methods. The Triple A mutant has a slightly higher (15–25%) level of cell surface expression than cysless WT P-gp, as shown by staining with MRK-16, a monoclonal antibody for P-gp (Figure 7A and Table 5). Total expression is also slightly higher than cysless WT P-gp (Figure 7C). Therefore the selected mutations at the drug-binding pocket did not affect the normal synthesis, folding and trafficking of the transporter. Using the conformation-sensitive antibody UIC2, the Triple A mutant also exhibited increased detection at the cell surface compared to cysless WT (Figure 7B).

3.5. Triple A mutant and Y953A displays decreased potency for reversal of NBD cyclosporine A transport by zosuquidar, tariquidar and elacridar

The transport function was determined by accumulation assays using various fluorescent substrates, at steady-state conditions. Figures 7D–E show representative histograms for transport function of the Triple A mutant. It displayed decreased transport for most of the substrates tested, with the exception of NBD-cyclosporine A (NBDCsA), which was

transported at 70–80% of the level of cysless WT (which was taken as 100% for calculation purposes, see Table 5).

The NBDCsA was transported by the Triple A mutant to a significant extent (70–80% compared to cysless WT), so the effect of zosuquidar, tariquidar and elacridar to reverse NBDCsA transport was investigated. At a 50 nM concentration, able to inhibit the transport of NBDCsA in cysless WT P-gp, the three drugs failed to exhibit any inhibitory effect for the Triple A mutant (Figure 8). These data suggest that the switch effect observed in the ATP hydrolysis measurements is linked with the ability of these inhibitors to reverse transport of substrates by P-gp. To further explore if the failure to inhibit transport function of P-gp is because of the combined effect of the three point mutations or the dominant effect of a particular one, the single mutants Y307A, Q725A and Y953A were also expressed in HeLa cells using the BacMam baculovirus system. All the single mutants were expressed at the cell surface similar to cysless WT P-gp and the transport function was near normal for all substrates tested (Table 5). The effect of 50 nM zosuquidar and tariquidar on transport of NBDCsA by these single mutants was tested and both drugs reversed the NBDCsA transport by Y307A and Q725A mutants, while there was no reversal (inhibition) for Y953A as compared to cysless WT Pgp (Figure 9). Also, 50 nM elacridar was unable to reverse NBDCsA transport by Y953A mutant P-gp (Figure 9).

As all three drugs were poor reversal agents of NBDCsA transport by Triple A and Y953A, as compared to the cysless WT, this effect was further investigated using a range of concentrations of zosuquidar, tariquidar and elacridar to determine their apparent affinities for the mutant P-gps. The IC_{50} of zosuquidar for reversal of NBDCsA transport by Y953A could not be calculated, as only 20% inhibition was observed at the highest concentration (Figure 9D). The IC_{50} of zosuquidar for Triple A mutant and of tariquidar and elacridar for both Y953A and Triple A mutants vs. cysless WT was 30 to 250-fold higher (Table 6 and Figures 8 and 9). We also checked their dose-dependent effect on transport of rhodamine 123 (Rh123) by the Y953A mutant, to confirm that this reversal effect (inhibition of transport) was not substrate specific. It was observed that the fold change in IC_{50} for reversal of Rh123 transport by zosuquidar and tariquidar was comparable to that observed for NBDCsA transport (data not shown).

3.6. Molecular modeling studies

We carried out docking studies at the central cavity of P-gp to assess how the inhibitors bind in the drug-binding pocket and how they interact with the polar residues. As the molecular structure of human P-gp is not available, a homology model based on the X-ray structure of mouse P-gp was used for docking studies. The improved X-ray structure of mouse P-gp bound to the inhibitor QZ59-SSS [3] was used as a template for the model. The mouse P-gp (*mdr1a*) amino acid sequence is very similar to human P-gp (87% sequence identity and 94% similarity) and the drug-binding pocket is similar with the exception of 6–8 residues, at least in terms of the amino acid sequence [21]. The program AutoDock Vina [22] was used to predict possible binding modes of the ligands. The residues of the drug-binding pocket were set as flexible for the docking studies because drug binding is likely accomplished through an induced-fit mechanism [29, 30]. For a number of reasons, docking studies of P-

gp cannot predict the exact location of drug binding [21], and this is clearly reflected in the fact that the program finds many poses with similar docking scores. The 10 poses with the highest scores for zosuquidar, elacridar and tariquidar are shown in Figure 10. These poses represent possible binding modes, and we selected those that matched the experimental data. Contacts between the ligands and protein residues were analyzed for all poses and interactions with distances shorter than 4 Å were taken into consideration. Interestingly, we found that 4 out of the 10 highest score poses of zosuquidar interact with Y953 through H-bond and F978 interacts with Y953 in a T-shape aromatic-aromatic interaction. The H-bond is directed towards the fluorine groups of zosuquidar (Figure 11, top left panel). In the case of elacridar, two phenylalanine residues (F978 and F728) produce the switch effect on ATPase activity. In one of the highest score poses, F978 interacts with Y953 in a T-shape manner, as does for zosuquidar, and F728 interacts with Y310, also in a T-shape fashion. Both tyrosine residues (Y953 and Y310) form H-bonds with elacridar (Figure 11, top right panel). In the case of tariquidar, one of the poses also matches the experimental data, showing the structural duos F978-Y953 and F728-Y310 interact with the drug and Q725 and Y307 H-bond to it (Figure 11, lower panels).

Molecular modeling studies thus predicted that mutation Y310A should also produce a switch (from inhibition to stimulation) for the ATP hydrolysis activity by elacridar and tariquidar. The Y310A mutant was therefore expressed in High-Five insect cells and modulation of ATPase activity was determined. As predicted, we found that elacridar and tariquidar indeed stimulated the basal activity of the Y310A mutant by $13 \pm 6\%$ and $18 \pm 12\%$, while they inhibited the activity of cysless WT P-gp by $74 \pm 7\%$ and $63 \pm 8\%$, at $1 \mu\text{M}$ and $0.125 \mu\text{M}$ drug concentrations, respectively (Tables 3 and 4).

Discussion

In P-gp, substrates and modulators bind in the transmembrane region, 50–60 Å from the site where ATP hydrolysis takes place, and the coordination of these two events remains poorly understood at the molecular level. Ligand-protein interactions at the drug-binding pocket of P-gp are expected to consist of van der Waals and hydrogen-bond (H-bond) interactions. The H-bonds are the most electrostatic because no charge residues are present in the drug-binding pocket to form ionic bonds [3, 5]. Although it is very difficult to estimate the strength of the non-covalent bonds between a particular drug and a protein, H-bonds are expected to be the stronger interactions, approximately 1–3 Kcal/mol, in comparison with less than 1.0 Kcal/mol estimated for van der Waals interactions [31].

The possibility of H-bond formation between the drugs and P-gp was investigated by mutation of polar residues of the drug-binding pocket. Previous studies showed that residues Y307, Q725 and Y953 are part of the large drug-binding pocket of P-gp [21, 24, 25]. These residues are conserved among P-gps from mammalian and avian species. A Triple A mutant, Y307A (TM 5)/Q725A (TM7)/Y953A (TM11), was designed to address the question of how some of the compounds inhibit ATP hydrolysis and drug transport by P-gp. Surprisingly, we found that the drugs that inhibit the basal ATPase activity of P-gp switch to stimulation in the case of the triple A mutation. The screening of polar and non-polar residues at the drug-binding pocket for their effect on the modulation of drug-mediated ATPase activity upon

mutation yielded a pair of structural motifs that are clearly relevant for the mechanism of inhibition. One is composed of Y953 and F978 and the other Y310 and F728. Residues Y307 and Q725 were also found to be important specifically for the interaction of tariquidar with P-gp.

Four different approaches were used to bring together the different pieces of the puzzle: ATP hydrolysis measurements, photo-crosslinking with IAAP, transport data and molecular modeling. Figure 6 illustrates that the ATPase data matches very well with IAAP-photocrosslinking data. Zosuquidar inhibited both the basal ATPase activity and IAAP-labeling of cysless WT P-gp. In contrast (and illustrated at the opposite end of Figure 6), zosuquidar stimulated the ATPase activity of the Triple A mutant, yet it was unable to inhibit binding of IAAP to this mutant protein. Zosuquidar did not inhibit the IAAP labeling because it could not bind at the primary binding site of the Triple A mutant; instead it appears to interact with a secondary or alternate site with lower affinity, stimulating basal ATP hydrolysis. Between these two extreme behaviors, there are mutations such as F343C, which have no apparent effect. Evidently, zosuquidar can bind at the primary site of the F343C mutant with high affinity through an H-bond to Y953, thus inhibiting both IAAP labeling and ATPase activity to the same extent as observed with cysless WT P-gp. But many other mutations of both polar and non-polar residues noticeably modify the binding site, preventing the normal binding of zosuquidar. As a consequence, zosuquidar is unable to inhibit the binding of IAAP and to inhibit the ATPase activity to the extent of cysless WT P-gp.

We measured the ability of these inhibitors to reverse or inhibit transport of fluorescent substrates and concluded that there is a correlation between the inhibition of ATP hydrolysis and the inhibition of the transport function. The apparent affinities show clearly that the ability to produce both effects is in the same concentration range for all the drugs under study (see Figure 2 and Table 6). Therefore, drugs bind at the same site to produce both outcomes, consistent with the observation that ATP hydrolysis and drug transport by P-gp are closely linked [32].

We identified two structural motifs that are essential for the drug-mediated inhibition of ATP hydrolysis by P-gp. Both motifs are formed by a tyrosine held by a phenylalanine in a typical T-shape aromatic-aromatic interaction. One (Y953-F978) has been demonstrated to be essential for the zosuquidar-mediated inhibition of the ATPase activity of P-gp, while both motifs are essential for the larger molecules elacridar and tariquidar. These motifs are also present in the transmembrane region of a eukaryotic Pgp homolog from *Cyanidioschyzon merolae* (CmABCB1, Y358-F383 and Y358'-F138) [33]. Several mammalian P-gp substrates including rhodamine 123, verapamil, paclitaxel, vinblastine, and valinomycin were shown to stimulate its ATPase activity. It will be interesting to see whether zosuquidar, elacridar and tariquidar inhibit the ATPase activity of this transporter. The Ca-Ca distances between the tyrosine and phenylalanine residues for Y953-F978 and Y310-F728 are 9 Å and 10 Å, respectively. The T-shape arrangement found between Phe and Tyr for both motifs is favored at such distances (8–10 Å) [34]. Nonetheless, the X-ray structure is only a snapshot of a very dynamic protein and it is easy to imagine that the conformational change that accompanies drug binding, closing of the NBDs and drug

translocation can disrupt the motifs or even bring the residues closer together. The recent X-ray structures of mouse Pgp with bound QZ59-compounds (pdb codes 4Q9I, 4Q9J, 4Q9K, and 4Q9L) show one or both motifs disrupted upon ligand binding [5]. Interestingly, the homology model of human P-gp based on the X-ray structure of SAV1866 that resembles the closed conformation (post ATP hydrolysis) shows one motif completely disrupted (Y953-F978) and the other (Y310-F728) with the interaction transformed from T-shape to pi-stacking [35]. This structural information strongly suggests that these drugs lock the protein in a conformation before the outward opening of the transmembrane domains.

A majority of substrates and modulators stimulate the ATPase activity of P-gp but only a few compounds including zosuquidar, elacridar and tariquidar inhibit it. In general, the compounds that stimulate ATPase activity exhibit lower binding affinity compared to those that inhibit it. We suggest that screening for drugs that inhibit the basal ATPase activity can therefore be a reliable tool to identify modulators with high affinity for P-gp, and possibly for other ABC drug transporters.

Acknowledgments

We thank Joshua Sitler and Paola Uscanga for technical assistance with ATP hydrolysis measurements, Dr. Stewart Durell for assistance with the molecular modeling studies, and George Leiman for editorial assistance. We acknowledge Drs. Tanaji Talele (St. John's University, Queens, NY) and John Golin (The Catholic University of America, Washington DC) for critical review of the manuscript. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. This study utilized the high-performance computational capabilities of the Helix and Biowulf Systems at the National Institutes of Health, Bethesda, MD.

References

1. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annual Review of Pharmacology and Toxicology*. 1999; 39:361–98.
2. Chufan EE, Sim HM, Ambudkar SV. Molecular Basis of the Polyspecificity of P-Glycoprotein (ABCB1): Recent Biochemical and Structural Studies. *Adv Cancer Res*. 2015; 125:71–96. [PubMed: 25640267]
3. Li JZ, Jaimes KF, Aller SG. Refined structures of mouse P-glycoprotein. *Protein Science*. 2014; 23:34–46. [PubMed: 24155053]
4. Jin MS, Oldham ML, Zhang QJ, Chen J. Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. *Nature*. 2012; 490:566. [PubMed: 23000902]
5. Szewczyk P, Tao H, McGrath AP, Villaluz M, Rees SD, Lee SC, et al. Snapshots of ligand entry, malleable binding and induced helical movement in P-glycoprotein. *Acta Crystallogr D Biol Crystallogr*. 2015; 71:732–41. [PubMed: 25760620]
6. Dawson RJP, Locher KP. Structure of a bacterial multidrug ABC transporter. *Nature*. 2006; 443:180–5. [PubMed: 16943773]
7. Polli JW, Wring SA, Humphreys JE, Huang LY, Morgan JB, Webster LO, et al. Rational use of in vitro P-glycoprotein assays in drug discovery. *Journal of Pharmacology and Experimental Therapeutics*. 2001; 299:620–8. [PubMed: 11602674]
8. Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM, et al. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. *Cancer Res*. 1996; 56:4171–9. [PubMed: 8797588]
9. Sparreboom A, Planting AS, Jewell RC, van der Burg ME, van der Gaast A, de Bruijn P, et al. Clinical pharmacokinetics of doxorubicin in combination with GF120918, a potent inhibitor of MDR1 P-glycoprotein. *Anticancer Drugs*. 1999; 10:719–28. [PubMed: 10573204]

10. Fox E, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. *Expert Review of Anticancer Therapy*. 2007; 7:447–59. [PubMed: 17428165]
11. Shukla S, Wu CP, Ambudkar SV. Development of inhibitors of ATP-binding cassette drug transporters - present status and challenges. *Expert Opinion on Drug Metabolism & Toxicology*. 2008; 4:205–23. [PubMed: 18248313]
12. Hendriks JJ, Lagas JS, Wagenaar E, Rosing H, Schellens JH, Beijnen JH, et al. Oral co-administration of elacridar and ritonavir enhances plasma levels of oral paclitaxel and docetaxel without affecting relative brain accumulation. *Br J Cancer*. 2014; 110:2669–76. [PubMed: 24781280]
13. Kapoor K, Bhatnagar J, Chufan EE, Ambudkar SV. Mutations in Intracellular Loops 1 and 3 Lead to Misfolding of Human P-glycoprotein (ABCB1) That Can Be Rescued by Cyclosporine A, Which Reduces Its Association with Chaperone Hsp70. *Journal of Biological Chemistry*. 2013; 288:32622–36. [PubMed: 24064216]
14. Shukla S, Schwartz C, Kapoor K, Kouanda A, Ambudkar SV. Use of Baculovirus BacMam Vectors for Expression of ABC Drug Transporters in Mammalian Cells. *Drug Metabolism and Disposition*. 2012; 40:304–12. [PubMed: 22041108]
15. Sauna ZE, Muller M, Peng XH, Ambudkar SV. Importance of the conserved Walker B glutamate residues, 556 and 1201, for the completion of the catalytic cycle of ATP hydrolysis by human P-glycoprotein (ABCB1). *Biochemistry*. 2002; 41:13989–4000. [PubMed: 12437356]
16. Ramachandra M, Ambudkar SV, Chen D, Hrycyna CA, Dey S, Gottesman MM, et al. Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry*. 1998; 37:5010–9. [PubMed: 9538020]
17. Kerr KM, Sauna ZE, Ambudkar SV. Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in Human P-glycoprotein. Evidence for ADP release as the rate-limiting step in the catalytic cycle and its modulation by substrates. *J Biol Chem*. 2001; 276:8657–64. [PubMed: 11121420]
18. Sauna ZE, Ambudkar SV. Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:2515–20. [PubMed: 10716986]
19. Ambudkar SV. Drug-stimulatable ATPase activity in crude membranes of human MDR1-transfected mammalian cells. *Methods Enzymol*. 1998; 292:504–14. [PubMed: 9711578]
20. Singh S, Prasad NR, Chufan EE, Patel BA, Wang YJ, Chen ZS, et al. Design and synthesis of human ABCB1 (P-glycoprotein) inhibitors by peptide coupling of diverse chemical scaffolds on carboxyl and amino termini of (S)-valine-derived thiazole amino acid. *J Med Chem*. 2014; 57:4058–72. [PubMed: 24773054]
21. Chufan EE, Kapoor K, Sim HM, Singh S, Talele TT, Durell SR, et al. Multiple Transport-Active Binding Sites Are Available for a Single Substrate on Human P-Glycoprotein (ABCB1). *Plos One*. 2013; 8:e82463. [PubMed: 24349290]
22. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. 2009; 31:455–61. [PubMed: 19499576]
23. Sanner MF. Python: A programming language for software integration and development. *Journal of Molecular Graphics & Modelling*. 1999; 17:57–61. [PubMed: 10660911]
24. Donmez Cakil Y, Khunweeraphong N, Parveen Z, Schmid D, Artaker M, Ecker GF, et al. Pore-exposed tyrosine residues of P-glycoprotein are important hydrogen-bonding partners for drugs. *Molecular Pharmacology*. 2014; 85:420–8. [PubMed: 24366667]
25. Loo TW, Clarke DM. Mapping the Binding Site of the Inhibitor Tariquidar that Stabilizes the First Transmembrane Domain of P-glycoprotein. *J Biol Chem*. 2015
26. Loo TW, Clarke DM. Membrane topology of a cysteine-less mutant of human P-glycoprotein. *J Biol Chem*. 1995; 270:843–8. [PubMed: 7822320]
27. Senior AE, al-Shawi MK, Urbatsch IL. The catalytic cycle of P-glycoprotein. *FEBS Lett*. 1995; 377:285–9. [PubMed: 8549739]

28. Scian M, Acchione M, Li M, Atkins WM. Reaction dynamics of ATP hydrolysis catalyzed by P-glycoprotein. *Biochemistry*. 2014; 53:991–1000. [PubMed: 24506763]
29. Loo TW, Bartlett MC, Clarke DM. Substrate-induced conformational, changes in the transmembrane segments of human P-glycoprotein - Direct evidence for the substrate-induced fit mechanism for drug binding. *Journal of Biological Chemistry*. 2003; 278:13603–6. [PubMed: 12609990]
30. Dolgih E, Bryant C, Renslo AR, Jacobson MP. Predicting Binding to P-Glycoprotein by Flexible Receptor Docking. *Plos Computational Biology*. 2011;7.
31. Lodish, HFa; Berk, Aa; Kaiser, Ca; Krieger, Ma; Bretscher, Aa; Ploegh, HLa, et al. *Molecular cell biology*. 7. International edition
32. Ambudkar SV, Cardarelli CO, Pashinsky I, Stein WD. Relation between the turnover number for vinblastine transport and for vinblastine-stimulated ATP hydrolysis by human P-glycoprotein. *Journal of Biological Chemistry*. 1997; 272:21160–6. [PubMed: 9261121]
33. Kodan A, Yamaguchi T, Nakatsu T, Sakiyama K, Hipolito CJ, Fujioka A, et al. Structural basis for gating mechanisms of a eukaryotic P-glycoprotein homolog. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111:4049–54. [PubMed: 24591620]
34. Chelli R, Gervasio FL, Procacci P, Schettino V. Stacking and T-shape competition in aromatic-aromatic amino acid interactions. *Journal of the American Chemical Society*. 2002; 124:6133–43. [PubMed: 12022848]
35. O'Mara ML, Tieleman DP. P-glycoprotein models of the apo and ATP-bound states based on homology with Sav1866 and MalK. *Febs Letters*. 2007; 581:4217–22. [PubMed: 17706648]

Figure 1. The affinity (K_m) for ATP is not significantly affected upon binding of zosuquidar or elacridar to P-gp

The Pgp-mediated ATP hydrolysis was measured in crude membranes of insect cells as described in Materials and methods. The figure compares the kinetic behavior of cystless WT P-gp (a) in the absence (top curve) and in the presence of different concentrations of zosuquidar (15 nM, 25 nM and 60 nM, from top to bottom); and (b) in the absence (top curve) and in the presence of elacridar (50 nM, 100 nM and 500 nM, from top to bottom). The data points were fit with the Michaelis-Menten equation, using Prism version 6.01, and the error bars denote the standard deviations. The values of K_m for ATP are given in the figure.

Figure 2. Drug-mediated inhibition switches to stimulation upon mutation of polar residues of the drug-binding pocket of P-gp

At least three independent experiments, each with duplicate samples, were carried out for both cysless WT and the Triple A mutant with indicated compounds, and error bars represent the standard deviations. The data points were fit with a one-phase decay equation, using Prism version 6.01. Basal activity was taken as 100% activity, and activities lower than 100% indicate inhibition while activities higher than 100% demonstrate stimulation. In all panels, black curves represent cysless WT and grey curves Triple A (Y307A/Q725A/Y953A) mutant P-gp. The IC_{50} (inhibition) and EC_{50} (stimulation) values are given in the figure. The chemical structure of the compounds is shown below the graphs. Zosuquidar exhibits the particular difluorocyclopropane group appended to a cycloheptane ring, while elacridar and tariquidar have the dimethoxy-isoquinolinyl-ethylphenyl-aminocarbonyl moiety in common.

Figure 3. Mutation at the residue Y953 dominates the switch effect (inhibition to stimulation) on ATP hydrolysis of Triple A (Y307A/Q725A/Y953A) mutant P-gp, by zosuquidar and elacridar Mutations of the polar residues Y307, Q725 and Y953 to alanine, cysteine and phenylalanine (Y953F only) were tested for their effect on the modulation of basal ATPase activity of P-gp by drugs. Vanadate-sensitive P-gp-mediated ATP hydrolysis was measured as described in Materials and methods. Basal activity of cysless WT and mutant P-gps was taken as zero, inhibition was calculated as percentage of the basal activity and shown with downward bars (negative values), while stimulation was calculated as percentage of the basal activity and shown with upward bars (positive values). Bars are colored black for cysless WT or triple A (Y307A/Q725A/Y953A) while they are grey for the single mutants (Y307A/C, Q725A/C, Y953A/C/F). At least three experiments were carried out with duplicate samples for each mutant with indicated compounds, and errors bars denote the standard deviations. Additional data on the modulatory effect at different drug concentrations is given in Table 2–4.

Figure 4. Substitution of selected phenylalanine residues with cysteine also produces the switch effect (from inhibition to stimulation)

Substitution of phenylalanine and other non-polar residues of the drug-binding pocket of P-gp to cysteine were tested for their effect on the modulation of basal ATPase activity by zosuquidar (0.5 μ M), elacridar (1 μ M) and tariquidar (0.125 μ M). Basal activity of the mutants was taken as zero, inhibition was calculated as a percentage of the basal activity and shown with downward bars (negative values), while stimulation was calculated as a percentage of the basal activity and shown with upward bars (positive values). Error bars denote standard deviation ($n > 3$). Additional data on the modulatory effect at different drug concentrations is given in Tables 2–4.

Figure 5. Effect of drugs on the photo-crosslinking of cysless WT and triple A (Y307A/Q725A/Y953A) mutant P-gp with IAAP

Crude membranes expressing cysless WT or mutant P-gp (60–80 μ g protein) were treated with the indicated concentrations of drug in 100 μ L buffer containing 50 mM MES-Tris pH 6.8 for 10 min at 37°C. Samples were then photo-crosslinked with 4–6 nM IAAP at 4°C as described in Materials and methods. IAAP-labeling of the cysless WT and Triple A mutant P-gp with no addition of drug was taken as 100% labeling. Both panels show a representative autoradiogram (at the top) and the quantification of the IAAP-labeling (at the bottom). Lane 1, control (DMSO solvent); 2, 1 μ M zosuquidar; 3, 1 μ M elacridar; and 4, 1 μ M tariquidar. Data represent the mean $\pm$ the standard deviations for $n=3$. Left panel, cysless WT; right panel, Triple A (Y307A/Q725A/Y953A) mutant.

Figure 6. Correlation of effects of zosuquidar on ATPase activity and IAAP-labeling of cysless WT and mutant P-gps

In the upper panel of the figure is shown the modulation of the basal ATPase activity of mutant P-gps by 0.5 μ M zosuquidar. Basal activity of the mutants was taken as zero, and inhibition or stimulation was calculated as percentage of the basal activity. The inhibition is shown with downward bars (negative values), while the stimulation is shown as upward bars (positive values). Vanadate-sensitive Pgp-mediated ATP hydrolysis was measured as described in Materials and methods. In the lower panel of the figure is shown the effect of zosuquidar (at different concentrations) on the photo-crosslinking of mutant P-gps with IAAP. Representative autoradiograms are shown. Photo-crosslinking experiments were carried out as described in the legend of Figure 5. The percentage of inhibition with 0.5 μ M zosuquidar (grey font) is given at the bottom of each autoradiogram.

Figure 7. Triple A (Y307A/Q725A/Y953A) mutant P-gp BacMam baculovirus-transduced HeLa cells exhibit normal cell surface expression but aberrant transport function for various substrates

(A) Triple-A mutant P-gp virus-transduced cells (250,000) were incubated with monoclonal antibody MRK-16 (1 μ g/100,000 cells) for 60 min at 37°C followed by incubation with FITC-conjugated anti-mouse IgG2a secondary antibody. The cell surface localization was detected with the green fluorescence detector. The dark grey histogram represents the cell surface expression of Cysless WT and is taken as 100 % (Table 5). The light grey trace marks the cell surface levels of the Triple A mutant P-gp. The black trace depicts labeling with the IgG2a isotype control. (B) Triple A mutant virus-transduced HeLa cells were incubated with conformation-sensitive UIC2 antibody (2 μ g/100,000 cells) in the absence and presence of 20 μ M CsA, for 30 min at 37°C followed by incubation with FITC-conjugated anti-mouse IgG2a secondary antibody. The histogram represents detection with UIC2 for Cysless WT when carried out both in the absence (trace with *) and presence of 20 μ M CsA (trace with #), while the traces that represent the cell surface levels for Triple A mutant in the absence and presence of 20 μ M CsA overlap each other (traces with ^). (C) Western Blot shows the levels of total P-gp in the HeLa cell lysates transduced with Cysless WT or Triple A mutant P-gp. Cell lysates (3.5 μ g protein/lane) were run on a 7% Tris-acetate gel and the immunoblot was developed with C219 antibody. The arrow and arrowhead show the position of mature and immature P-gp bands, respectively. (D, E) Typical histograms show transport function of Triple A mutant using JC-1 and NBDCsA fluorescent substrates. The inactive EQ (E556Q/E1201Q) mutant P-gp was used to determine total accumulation (no efflux). The figure shows typical histograms from one representative experiment, which was done independently at least three times.

Figure 8. Triple A (Y307A/Q725A/Y953A) mutant P-gp displays decreased reversal of transport function by zosuquidar, tariquidar and elacridar

The upper panels (A–C) show histograms for the accumulation of NBDCsA by the cysless WT and triple A (Y307A/Q725A/Y953A) mutant P-gps. The histograms also show that NBDCsA transport by triple A mutant (brown curves) is not reversed by the presence of 50 nM zosuquidar (A), 50 nM tariquidar (B) or 50 nM elacridar (C), while these compounds at 50 nM completely reverse transport by cysless WT P-gp (curves with maximum fluorescence intensity). The lower panels (D–F) show the reversal of NBDCsA efflux by zosuquidar, tariquidar and elacridar at various concentrations. The transport of NBDCsA in the absence of any inhibitor was taken as 100% for both cysless WT and the Triple A mutant, respectively, and the extent of transport in the presence of the given concentration of an inhibitor was calculated with respect to it. Data points were plotted as the mean + SD ($n=3$) using GraphPad Prism 6.0. The IC₅₀ values are reported in Table 6.

Figure 9. Y953A mutant P-gp displays significantly decreased reversal of transport function by zosuquidar, tariquidar and elacridar

The upper panels (A–C) show the histograms for the transport of NBDCsA by the cysless WT and Y953A mutant P-gps. The histograms also show the effect of 50 nM zosuquidar (A), 50 nM tariquidar (B) or 50 nM elacridar (C) on reversal of NBDCsA transport by Y953A mutant P-gp, and by cysless WT P-gp (traces with maximum fluorescence intensity). The lower panels (D–F) show the effect of zosuquidar, tariquidar and elacridar at indicated concentrations on reversal of NBDCsA transport by cysless WT and Y953A mutant P-gp. The transport of NBDCsA in the absence of any inhibitor was taken as 100% for both cysless WT and Y953A mutant, respectively, and the percent of transport in the presence of the different inhibitors was calculated with respect to it. Data points are plotted as the mean + SD ($n=3$). The values of IC₅₀ (compound concentration that produces 50% inhibition of NBDCsA transport in HeLA cells expressing cysless WT or Y953A mutant P-gp) are given in the figure. The IC₅₀ value of zosuquidar could not be calculated, as a maximum 20% inhibition was observed at the highest concentration. The data was plotted and IC₅₀ values calculated using GraphPad Prism 6.0.

Figure 10. Docking of zosuquidar, elacridar and tariquidar in the binding pocket of human Pgp Exhaustive ligand docking in the homology model of human Pgp based on mouse P-gp structure 4M2T.pdb [3] was carried out using a receptor grid centered at the position of the QZ59-RRR molecule (shown as light grey sticks in the right lower panel), with flexible side-chains covering an ample space (see Materials and methods for the list of residues selected to be treated as flexible) and a search box of dimensions $40\text{\AA} \times 35\text{\AA} \times 35\text{\AA}$. The first 10 modes with the highest docking scores (Kcal/mol, given in the figure) were clustered and shown as black sticks model in the corresponding panels. The original positions of QZ59-RRR (light grey) and QZ59-SSS (dark grey) molecules are shown in the right lower panel as stick models, using the original coordinates from crystal structure 4M2S/4M2T.pdb, respectively. Helices 4 and 5 are not shown for clarity. The figure was prepared with PyMOL 1.5.

Figure 11. Tyrosine-phenylalanine structural motifs in the drug-binding pocket of human P-gp are critical for interaction with zosuquidar, elacridar and tariquidar

Zosuquidar, elacridar and tariquidar were docked in the central cavity of the homology model of human P-gp based on the improved mouse P-gp X-ray structure (4M2T.pdb, [3]). The panels show selected poses that match the experimental data. For zosuquidar, Y953 is H-bonded to a fluorine group of the drug and F978 is in T-shape aromatic-aromatic contact [34] with Y953. For elacridar, Y953 and Y310 are H-bonded to the drug with F978 and F728 in T-shape aromatic-aromatic interaction with the Y953 and Y310 residues, respectively. For tariquidar, side A shows the Y953-F978 structural motif with Y953 H-bonded to the drug and side B shows the Y310-F728 structural motif with Y310 H-bonded to the drug. Side B also shows Q725 and Y307 H-bonded to tariquidar. Zosuquidar, elacridar, tariquidar and selected residues are shown in stick models, the rest of the surrounded residues are shown as grey balls at the position of the α -carbon. Color code: C(residues)=green; C(drug-aliphatic)=cyan; C(drug-aromatic)=white; O=red; N=blue and F=light blue. H-bonds and aromatic-aromatic interactions are indicated as dashed lines. The figures were prepared with PyMOL 1.5.

Table 1
Substrates and modulators that stimulate the basal ATPase activity of cysless WT also stimulate the activity of triple A (Y307A/Q725A/Y953A) mutant P-gp

The percentage stimulation of the basal ATPase activity of cysless WT and mutant P-gps by the given drugs at indicated concentrations are shown. ATP hydrolysis in the presence and absence of the indicated drugs was determined as described in Materials and methods. Values represent the average of two experiments.

Drug	Concentration (μM)	ATP hydrolysis, % stimulation	
		Cysless WT	Y307A/Q725A/Y953A
Verapamil	1	31%	9%
	5	44%	25%
	25	52%	107%
Rhodamine 123	0.1	52%	179%
	1	46%	156%
Daunorubicin	1	54%	76%
	10	41%	35%
Rapamycin	0.1	25%	44%
	1	24%	92%
Bodipy FL-prazosin	1	28%	41%
	20	23%	47%
Valinomycin	10	100%	310%
QZ59S-SSS	1	84%	87%
Nilotinib	1	50%	73%
Erlotinib	50	79%	166%
Cisflupentixol	25	73%	206%
Hoechst 33342	1	43%	63%
GPV001 (propafenone)	10	92%	82%
GPV574 (propafenone)	10	83%	128%

Table 2
Modulation of the basal ATPase activity of cysless WT and mutant P-gps by zosuquidar, at two different concentrations

The percentage modulation is reported in black with a downward pointing arrow (↓) for inhibition, and in red with an upward pointing arrow (↑) for stimulation. The results were sorted as “no significant change”, “slight change” and “drastic change” depending on how different the ATPase activity was in comparison with the basal activity. ATP hydrolysis was determined as described in Materials and methods. Values represent mean $\pm$ SD (standard deviation) for $n = 3$.

Mutation(s)	Zosuquidar	
	0.5 μ M	5 μ M
Cysless WT (control)	↓83 $\pm$ 1%	↓94 $\pm$ 5%
No significant change		
F343C	↓86 $\pm$ 1%	↓91 $\pm$ 2%
F335C	↓78 $\pm$ 1%	↓84 $\pm$ 1%
Y310F	↓74 $\pm$ 3%	↓79 $\pm$ 4%
M949C	↓61 $\pm$ 10%	↓72 $\pm$ 7%
Y950A	↓67 $\pm$ 5%	↓64 $\pm$ 2%
L975C	↓69 $\pm$ 2%	↓86 $\pm$ 2%
Y307C	↓53 $\pm$ 2%	↓73 $\pm$ 10%
Q725C	↓52 $\pm$ 10%	↓65 $\pm$ 7%
Y307C/Q725C/V982C	↓55 $\pm$ 4%	↓66 $\pm$ 11%
V982C	↓46 $\pm$ 3%	↓57 $\pm$ 4%
Slight change		
Y307A	↓47 $\pm$ 5%	↓57 $\pm$ 5%
Q725A	↓34 $\pm$ 15%	↓53 $\pm$ 10%
F728C	↓35 $\pm$ 8%	↓48 $\pm$ 8%
I306C	↓32 $\pm$ 12%	↓62 $\pm$ 11%
Y310A	↓10 $\pm$ 8%	↓48 $\pm$ 5%
Drastic change		
Y953F	↑10 $\pm$ 7%	↓18 $\pm$ 14%
F978C	↑19 $\pm$ 5%	↓42 $\pm$ 6%
Y307C/Q725C/V982C /Y953A	↑10 $\pm$ 4%	↑11 $\pm$ 4%
Y953A	↑21 $\pm$ 7%	↑24 $\pm$ 9%
Y953C	↑37 $\pm$ 8%	↑28 $\pm$ 8%
Y307A/Q725A/Y953A	↑71 $\pm$ 5%	↑65 $\pm$ 9%

Table 3
Modulation of the basal ATPase activity of cysless WT and mutant P-gps by elacridar, at two different concentrations

See Table 2 for experimental details and further explanation. Values represent mean $\pm$ SD (standard deviation) for $n = 3$.

Mutation(s)	Elacridar	
	1 μ M	10 μ M
Cysless WT (control)	$\downarrow 74 \pm 7\%$	$\downarrow 78 \pm 3\%$
No significant change		
F343C	$\downarrow 75 \pm 2\%$	$\downarrow 84 \pm 1\%$
L975C	$\downarrow 65 \pm 3\%$	$\downarrow 64 \pm 4\%$
Slight change		
Y950A	$\downarrow 51 \pm 8\%$	$\downarrow 57 \pm 3\%$
I306C	$\downarrow 49 \pm 13\%$	$\downarrow 46 \pm 2\%$
Y310F	$\downarrow 41 \pm 5\%$	$\downarrow 43 \pm 2\%$
V982C	$\downarrow 32 \pm 19\%$	$\downarrow 37 \pm 9\%$
Y307C	$\downarrow 32 \pm 8\%$	$\downarrow 34 \pm 6\%$
F335C	$\downarrow 31 \pm 2\%$	$\downarrow 60 \pm 1\%$
M949C	$\downarrow 30 \pm 6\%$	$\downarrow 40 \pm 13\%$
Y307C/Q725C/V982C	$\downarrow 17 \pm 5\%$	$\downarrow 38 \pm 4\%$
Y307A	$\downarrow 17 \pm 14\%$	$\downarrow 12 \pm 8\%$
Q725A	$\downarrow 17 \pm 6\%$	$\downarrow 13 \pm 9\%$
Y953F	$\downarrow 5 \pm 9\%$	$\downarrow 17 \pm 7\%$
Q725C	$\downarrow 1 \pm 17\%$	$\downarrow 31 \pm 4\%$
Drastic change		
F728C	$\uparrow 8 \pm 9\%$	$\downarrow 19 \pm 9\%$
Y310A	$\uparrow 13 \pm 6\%$	$\uparrow 13 \pm 8\%$
F978C	$\uparrow 31 \pm 9\%$	$\uparrow 10 \pm 8\%$
Y307C/Q725C/V982C /Y953A	$\uparrow 43 \pm 7\%$	$\downarrow 3 \pm 3\%$
Y953C	$\uparrow 76 \pm 10\%$	$\downarrow 25 \pm 2\%$
Y953A	$\uparrow 83 \pm 4\%$	$\downarrow 18 \pm 4\%$
Y307A/Q725A/Y953A	$\uparrow 81 \pm 6\%$	$\uparrow 58 \pm 11\%$

Table 4
Modulation of the basal ATPase activity of cysless WT and mutant P-gps by tariquidar,
at two different concentrations

See Table 2 for experimental details and further explanation. Values represent mean $\pm$ SD (standard deviation) for $n = 3$.

Mutation(s)	Tariquidar	
	0.125 μ M	1.25 μ M
Cysless WT (control)	$\downarrow 63 \pm 8\%$	$\downarrow 70 \pm 13\%$
No significant change		
F343C	$\downarrow 75 \pm 2\%$	$\downarrow 82 \pm 2\%$
M949C	$\downarrow 55 \pm 7\%$	$\downarrow 58 \pm 5\%$
L975C	$\downarrow 52 \pm 6\%$	$\downarrow 65 \pm 5\%$
I306C	$\downarrow 44 \pm 11\%$	$\downarrow 67 \pm 11\%$
Y950A	$\downarrow 42 \pm 16\%$	$\downarrow 60 \pm 3\%$
Slight change		
Y310F	$\downarrow 28 \pm 1\%$	$\downarrow 40 \pm 4\%$
Y953F	$\downarrow 25 \pm 9\%$	$\downarrow 40 \pm 9\%$
F335C	$\downarrow 24 \pm 8\%$	$\downarrow 65 \pm 2\%$
Y307C	$\downarrow 20 \pm 5\%$	$\downarrow 35 \pm 3\%$
V982C	$\downarrow 11 \pm 3\%$	$\downarrow 32 \pm 18\%$
Drastic change		
Y307C/Q725C/V982C	$\downarrow 1 \pm 4\%$	$\downarrow 20 \pm 13\%$
Q725C	$\uparrow 1 \pm 7\%$	$\downarrow 4 \pm 14\%$
Y307A	$\uparrow 2 \pm 4\%$	$\downarrow 9 \pm 2\%$
Q725A	$\uparrow 3 \pm 10\%$	$\downarrow 10 \pm 5\%$
Y953C	$\uparrow 3 \pm 7\%$	$0 \pm 8\%$
Y307C/Q725C/V982C /Y953A	$\uparrow 16 \pm 8\%$	$\downarrow 4 \pm 4\%$
Y953A	$\uparrow 18 \pm 1\%$	$\uparrow 12 \pm 5\%$
Y310A	$\uparrow 18 \pm 12\%$	$\uparrow 21 \pm 7\%$
F978C	$\uparrow 23 \pm 5\%$	$\uparrow 32 \pm 5\%$
F728C	$\uparrow 27 \pm 4\%$	$\uparrow 9 \pm 8\%$
Y307A/Q725A/Y953A	$\uparrow 42 \pm 9\%$	$\uparrow 90 \pm 9\%$

Table 5
The cell surface expression and transport function of single and triple mutant P-gps

The values indicate the range of cell surface expression and steady-state accumulation of fluorescent substrates in HeLa cells expressing mutant P-gps compared to cysless WT. The cell surface expression of P-gps in BacMam virus-transduced HeLa cells was determined by flow cytometry using MRK-16 antibody. The expression of cysless WT was taken as 100% and the level of expression of single and triple mutant P-gps were calculated compared to cysless WT. To assess the transport function of variant P-gps, accumulation of various fluorescent substrates including calcein-AM (CalAM, 0.5 μ M), NBD-cyclosporine A (NBDCsA, 0.5 μ M), rhodamine 123 (Rh123, 1.3 μ M), daunorubicin (dauno, 0.5 μ M) bodipy-prazosin (BD-PRA, 0.5 μ M), bodipy-vinblastine (BD-VBL, 0.5 μ M) and JC-1 (0.5 μ M), was determined as described in Materials and methods. The transport function of cysless WT was taken as 100% and the transport by other variants was calculated with respect to it. The values from three or more experiments are reported.

Mutation(s)	Cell Surface Expression (%) ^a	Transport Function (%) ^b						
		Cal AM	NBD CsA	Rh123	Dauno	BD-PRA	BD-VBL	JC-1
Y307A	80–90	90–100	80–90	90–100	80–90	70–80	70–80	90–100
Q725A	90–100	90–100	90–100	90–100	90–100	90–100	90–100	90–100
Y953A	90–100	90–100	90–100	90–100	75–85	75–85	90–100	90–100
Y307A/Q725A/Y953A	120–140	40–50	70–80	20–30	20–30	0–10	30–40	10–20

^aThe cell surface expression of cysless WT P-gp was taken as 100%.

^bThe steady-state transport of indicated fluorescent substrate by cysless WT P-gp was taken as 100%.

Table 6

Apparent binding affinities of drugs decrease significantly upon mutation of polar residues in the drug-binding pocket of P-gp.

Compound	Inhibition of NBDCsA Transport		
	Cysless WT	Triple A mutant ^a	Fold Decrease
	IC ₅₀ (nM) ^b	IC ₅₀ (nM) ^b	
Zosuquidar	8 ± 1	243 ± 46	30
Tariquidar	7 ± 1	203 ± 31	29
Elacridar	7 ± 2	1,738 ± 586	250

^aTriple A mutant, Y307A/Q725A/Y953A

^bIC₅₀, compound concentration that produces 50% inhibition of NBDCsA transport in HeLA cells expressing cysless WT or mutant P-gp (values [mean ± SD] calculated from data in Figure 5–D, E, and F).