Dynamic Mechanisms Of Multidrug Resistance In Human Cancers And Gram Negative Pathogens

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DYNAMIC MECHANISMS OF MULTIDRUG RESISTANCE IN HUMAN CANCERS AND GRAM-NEGATIVE PATHOGENS.

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DYNAMIC MECHANISMS OF MULTIDRUG RESISTANCE IN HUMAN CANCERS AND GRAM-NEGATIVE PATHOGENS.

A Dissertation Presented to the Graduate Faculty of the

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Southern Methodist University

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for the degree of

Doctor of Philosophy

with a

Major in Molecular and Cellular Biology

by

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Why do they call it a pelican?
This dissertation focuses upon dynamic agents of multidrug resistance (MDR). We used a combination of in silico and in vitro techniques to investigate two membrane transporters that confer MDR – P-glycoprotein, which confers MDR in human cancers, and MtrD, which confers MDR in Neisseria gonorrhoeae. Inhibitors targeting both proteins have tremendous potential for use as co-therapeutics in the treatment of multidrug resistant cancers, or of multidrug resistant infections. However, previously identified inhibitors of P-gp have failed clinical trials due to off-target effects and associated toxicities. Furthermore, the molecular mechanism of antibiotic transport by MtrD is poorly understood, and this dearth of knowledge obfuscates efforts to target MtrD in drug discovery screens. For these reasons, inhibitors targeting either P-gp or MtrD are not available for clinical use.

In this work, we used rigorous and robust computational techniques to investigate P-gp and MtrD. Our studies employed free and biased all atom Molecular Dynamics simulations to study the conformational and transport dynamics of these MDR-critical proteins. We then used massively parallel molecular docking experiments to screen millions of compounds prior to purchasing them for testing in the laboratory. Finally, to test our computationally-derived hypotheses, we employed a suite of cell-based and biophysical assays; these included MTT cell
viability assays, Confocal Microscopy, Fluorescence Accumulation Assays, and LC-MS/MS accumulation assays.

In this work, we contributed to the understanding of how MtrD effluxes its antibiotic substrates, thereby rendering them ineffective as treatments for *N. gonorrhoeae*. Our data identify new areas of MtrD to target in drug discovery efforts. Furthermore, we used a massively parallel virtual drug screening program to select compounds for testing as P-gp inhibitors and tested those compounds against five different human cell lines *in vitro*. We report a 13% hit rate for P-gp inhibitors, a massive improvement over other virtual-assisted screens. As our last project, we used MD simulations and cell-based assays to demonstrate that P-gp can efflux the Alzheimer’s associated Aβ peptides. This project significantly expands the substrate profile of this already promiscuous transporter.
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\[ MLP_k = \sum_{i=1}^{N} F_i \cdot f(d_{ik}) \]

\[ LI = \frac{\sum MLP^+}{\sum MLP^+ + |\sum MLP^-|} \times 100 \]

\[ \% \text{Hydration of SASA} = \frac{\# H_2O \text{ at frame N}}{\# H_2O \text{ of Complete Hydration Layer}} \]

\[ \% \text{Viability} = 100 \times \frac{\text{Fluorescence}_{\text{experimental}} - \text{Fluorescence}_{\text{background}}}{\text{Fluorescence}_{\text{DMSO-treated}} - \text{Fluorescence}_{\text{background}}} \]

\[ \text{Ratio Analyte / Internal Standard (IS)} = \frac{\text{Analyte Peak Area (counts)}}{\text{IS Peak Area (counts)}} \]

\[ \text{Normalized Ratio} = \frac{\text{Ratio of Analyte / IS}}{\text{Sample Content (mg / ml)}} \]

\[ \text{Mean Ratio} = \frac{\sum_{i=1}^{n} \text{Ratio}_i}{n \text{ samples}} \]

\[ \Delta \text{DAU Fluorescence} = \frac{\text{Fluorescence of Compound + DAU treated cells}}{\text{Fluorescence of DAU treated cells}} \]

\[ \text{Integrated Density}_{\text{area}} = \text{Sum(value each pixel)} \times 0.024329 \mu^2 \text{pixel}^{-1} \]
\[ Mean \text{ Intensity} = \frac{\text{Integrated Density}_{\text{area}}}{N_{\text{pixels in area}}} \]

Corrected Mean Intensity_{\alpha\beta} = Mean \text{ Intensity}_{\alpha\beta} - Mean \text{ Intensity}_{\text{background}}
Dedicated to Keanu, Finn, and James.
INTRODUCTION

This work explores dynamic mechanisms of multidrug resistance (MDR) in humans and in gram-negative bacteria. MDR contributes to poor clinical outcomes and has been observed in both human cancers [1] and in obligate human pathogens, specifically gram-negative bacteria [2]. As a mechanism of MDR, organisms commonly overexpress dynamic membrane efflux transporters. These polyspecific enzymes export chemotherapeutics, antibiotics, and other xenobiotics out of the cell, thereby rendering them ineffective. Inhibition of multidrug efflux transporters can re-sensitize MDR organisms to therapeutics [3-6]; consequently, it is increasingly important to understand the mechanisms of these dynamic transporters.

Here we explore two agents of MDR: P-glycoprotein (P-gp, ABCB1) and the Multiple transferrable resistance protein D (MtrD, mtrD). P-gp confers MDR in human cancers [1], and MtrD confers MDR in Neisseria gonorrhoeae [7]. Both proteins export a variety of structurally and chemically diverse therapeutics and are considered clinically relevant targets for drug discovery [8, 9]. In Chapter 1, we discuss our investigation of MtrD using explicit, all-atom Molecular Dynamics (MD) simulations. In Chapter 2, we discuss our identification of novel P-gp inhibitors using a virtual-assisted drug discovery program and cell-based assays. Lastly, in Chapter 3, we provide evidence that significantly expands the substrate profile of P-gp using MD simulations, cell-based assays, and biophysical measurements. This work contributes significantly to the understanding of both P-gp and MtrD and lays the foundation for targeting both transporters in drug discovery efforts.
CHAPTER 1.

These data have been submitted for publication – PLOS Comp Bio, Ammerman et al. 2021.

1.1. Introduction

The gram-negative diplococcus *Neisseria gonorrhoeae* is responsible for the sexually transmitted infection (STI) Gonorrhea. Untreated gonococcal infections can result in severe reproductive health consequences, including but not limited to pelvic inflammatory disease, ectopic pregnancies, and infertility [10]. Gonorrhea spreads easily, and many infected individuals are asymptomatic and can spread the STI unknowingly [10, 11]. With approximately 1.14 million new cases reported each year in the United States (a 64% increase since 2014), Gonococcal infections are classified as an urgent health concern [10, 11]. Of these (reported) cases, approximately *half* are classified as drug resistant [11].

*N. gonorrhoeae* acquires antibiotic resistance relatively quickly. For example, in just seven years after its introduction in 2000 as a treatment for gonorrhea, the antibiotic Ciprofloxacin is no longer a recommended treatment [11]. In 2020, the U.S. Centers for Disease Control (CDC) stated that the antibiotic Ceftriaxone is the last recommended treatment for uncomplicated gonorrhea [10]. For multidrug resistant gonococcal infections, only one recommended treatment remains – combination treatment with Ceftriaxone and Azithromycin [2]. Unfortunately, in 2018 a strain of *N. gonorrhoeae* with resistance to both azithromycin and ceftriaxone was detected [12]. Thus, the options for treatment of gonorrhea are dwindling, and it
is increasingly important to understand and target the mechanisms that confer antibiotic resistance to this pathogen.

Gram negative pathogens like *N. gonorrhoeae* have evolved intricate mechanisms to overcome antimicrobial treatments [13]. Five multidrug efflux systems have been identified in *N. gonorrhoeae* – MtrCDE, MacAB, NorM, FarAB and MtrF – but the multiple transferrable resistance (Mtr)CDE system is the best characterized [7, 9, 14-20]. MtrCDE is a member of the Resistance-Nodulation Cell Division (RND) family of multidrug efflux systems [9]. RND systems assemble as tripartite protein complexes consisting of an efflux pump embedded in the inner membrane, a channel that passes through the outer membrane, and a periplasmic adaptor that connects the pump to the channel [13]. The MtrCDE system consists of the MtrD pump, the MtrC periplasmic adaptor, and the MtrE channel [19]. As member of the hydrophobic amphiphilic efflux (HAE) family of RND transporters, MtrCDE effluxes a variety of hydrophobic and amphiphilic substrates [9]. Overexpression of MtrCDE contributes significantly to clinical levels of antibiotic resistance in *N. gonorrhoeae*, and mutations in the drug binding region of MtrD can modulate resistance to a variety of antibiotics [7, 14-16, 18]. Additionally, it is thought that MtrCDE is the only RND efflux system expressed in *N. gonorrhoeae* [7]. Thus, the MtrCDE system is a promising target for the development of antimicrobial agents targeting gonococcal infections.

The MtrD efflux pump assembles as a homotrimer, and each protomer consists of a large periplasmic domain and 12 transmembrane helices (Figure 1.1.1A) [19, 20]. The periplasmic region of MtrD forms both the periplasmic cleft and the docking domain (Figure 1.1.1B). While the periplasmic cleft is responsible for the capture and extrusion of substrates, the docking domain interfaces with the MtrCE complex. The periplasmic cleft (residues 34-179, 272-327,
562-721, 809-862) is further divided into the Access Pocket (residues 562-721, 809-863), which consists of the PC1 (residues 562-666) and PC2 (residues 667-721, 809-862) domains, and the Deep Pocket (residues 34-179, 272-327), which consists of the PN1 (residues 34 to 133) and PN2 (residues 134 to 179, 272 to 327) domains (Figure 1.1.C). The Access and Deep Pockets also house the proximal and distal multidrug binding sites [17]. The Access and Deep Pockets are bisected by a flexible and conserved G-Loop (residues 610-616) (Figure 1.1.C) [7]. The G-Loop is thought to play a role in substrate filtration and orientation [7, 17, 20].
Figure 1.1.1. **The MtrD Efflux Pump from Neisseria gonorrhoeae.**
(A) The MtrD homotrimer with subunits colored orange, gray and blue. (B) An MtrD monomer with helices of the Access Pocket in yellow and the G-Loop in magenta. (C) The periplasmic cleft (residues 34-179, 272-327, 562-721, 809-862) viewed as if looking from the periplasm towards the inner membrane; helices of the Access Pocket in yellow, helices of the Deep Pocket in green, G-Loop (residues 610-616) in magenta. K823 and R714 may contribute to macrolide recognition (in orange sticks), and F612 and F610 may facilitate substrate selectivity [17]. (D) The Proton Relay Network. (E) The Access Pocket (residues 562-721, 809-863) viewed from the periplasm; PC2 (residues 667-721 and 809-862, and shaded yellow) closes during transport. (F) The Deep Pocket (residues 34-179, 272-327) viewed from the center; PN2 (residues 134 to 179 and 272 to 327, and shaded green) opens during transport. Stages of the transport cycle are labeled Access (also ‘Loose’), Binding (‘Tight’), Extrusion (‘Open’), and Intermediate with the corresponding crystal structure of the MtrD homologue AcrB (5NC5, 4DX5) or CmeB (5LQ3) in parentheses [20, 21].
The transmembrane helices of MtrD house the highly conserved Proton Relay Network (PRN) that utilizes the proton gradient across the inner membrane (Figure 1.1.D) [20]. Changes in the protonation state(s) of these residues are thought to facilitate vertical shearing motions of the TM helices, which correlate with peristaltic motions of the periplasmic cleft – thereby facilitating substrate movement [22]. Bulky substrates are thought to bind first at the proximal binding site in the Access Pocket, and then subsequently bind at the distal binding site in the Deep Pocket; extrusion then occurs through the funnel created by the docking domains of the homotrimer [22, 23]. The proximal (residues 79, 574, 669, 670, 714, and 823) and distal (residues 134, 136, 175, 176, 325, 570, 607, 623) sites are loosely defined, and their respective compositions are based upon the results of mutational studies (which also identified residues 714 and 823 as important for macrolide recognition) and upon crystal structures of MtrD homologues [17]. MtrD is thought to capture substrates from the periplasmic leaflet of the inner membrane, or from the periplasmic solvent itself [2]. Known transport substrates of MtrD include bile salts, antimicrobial peptides, dyes, β-lactams, and macrolides – the latter including both erythromycin and azithromycin [7, 9, 17].

To transport a substrate, MtrD is thought to undergo a series of conformational changes that primarily occur in the periplasmic cleft and transmembrane helices (Figure 1.1.E-F, Figure 1.1.2). Several distinct conformations of RND pumps have been identified by structural studies. In order, they are (1) ‘Access/Loose’, (2) ‘Binding/Tight’ (3) ‘Extrusion/Open’, and a fourth putative (4) ‘Resting/Intermediate’ conformation, which may be a transitional structure between the Extrusion and Access states (Figure 1.1.E,F) [17, 21]. Substrate-free MtrD adopts a symmetrical conformation with each protomer in the Access state [20]. Upon the binding of a transport substrate, the trimer adopts an asymmetric conformation with each protomer in one of
the conformational states – Access, Binding, or Extrusion [17]. The substrate is thought to move from the Access Pocket to the Deep Pocket during the transition from Access to Binding [23]. Each monomer of MtrD cycles sequentially from Access, to Binding, to Extrusion in a functional rotation mechanism [17]. The molecular characteristics of the periplasmic cleft contribute to the substrate specificity of MtrD, and some of these features are thought to change dynamically during transport [24]. However, it is unclear how these conformational changes result in the recognition and extrusion of substrates by MtrD.

Figure 1.1.2. **Modeling a Putative Catalytic Transport Cycle of MtrD.**
(A) The MtrD monomer viewed from the periplasm, with helices of the Access Pocket colored yellow, and the G-Loop in magenta. We show the structures of MtrD homologues, in order, that were used to model a putative efflux cycle with TMD simulations. The stage of transport – Access, Binding, Extrusion, Transition (Intermediate) is above the structures used; the PDB ID, the specific chain of the PDB, as well as the name of the source protein, are also shown. (B) The periplasmic cleft of MtrD viewed from the back, or from the center of the transporter. Helices of the Deep Pocket are colored green, the G-Loop is in magenta. These images are derived from the same structures as in (A) but rotated 180° and viewed from the back.
Therefore, to understand the process of substrate transport by MtrD, we simulated the interactions between wild-type MtrD and two antibiotics: azithromycin, a substrate of MtrD, and streptomycin, a non-substrate of MtrD [7, 9]. Using both free and directed Molecular Dynamics (MD) simulations, we modeled a putative efflux cycle using conformations of MtrD homologues [25]. In our Targeted Molecular Dynamics (TMD) simulations, small forces were applied to α-carbons of MtrD, but not to any other atoms in the system. Since substrates are thought to bind first at the proximal site in the periplasmic cleft [6], we tested two start sites within the proximal site for each ligand. We simulated each of the three protonation states of azithromycin that could occur at physiological pH ranges; it should be noted that only one protonation state of streptomycin should occur at physiological pH. We also tested the effect of including a fourth putative Intermediate conformation in the target sequence. To confirm our findings in these TMD simulations, we performed a 1.5 µs equilibrium simulation of azithromycin and MtrD. TMD simulations were performed with NAMD and CHARMM36 force fields, whereas the long time-scale simulation was performed with AMBER18 and the pmemd.cuda-DPFP molecular dynamics engine [26-29].

We observed the transport of azithromycin and the passive rejection of streptomycin by MtrD in TMD simulations. We found that the dynamic molecular landscape of the periplasmic cleft facilitates substrate discrimination, transport, and extrusion. Structures of the MtrD homologue AcrB and of MtrD<sub>CR103</sub>, a variant that confers elevated azithromycin resistance, suggest that macrolides bind to the distal binding site in the Deep Pocket [17, 30]. However, azithromycin was not observed in our TMD simulations to interact with residues of the distal site, but instead took an alternate transport pathway along PC2 and PN1, mediated by water and polar interactions. In TMD simulations, we also observed that the fourth putative conformation
(Intermediate) increases the total movement of azithromycin through the periplasmic cleft. In an unconstrained 1.5 µs MD simulation of azithromycin-bound MtrD in the Access conformation, azithromycin was observed to move past the G-Loop and into the Deep Pocket. We note that this movement, and any correlated conformational changes of MtrD, occurred in the absence of biasing forces or changes to the PRN. Lastly, to explore the role of the membrane fusion protein MtrC, we built a complete MtrCDE complex using known structures and molecular modeling.

Taken together, our data suggest that multiple pathways through the periplasmic cleft may exist — even for bulky macrolides like azithromycin. Our data indicate that MtrC may not actively contribute to the capture and extrusion of substrates. However, our simulations do suggest that a unique feature of MtrD, the TM9 helix and TM9-TM10 linker, might facilitate the capture of amphiphilic substrates from the periplasmic leaflet of the inner membrane. Our results support the hypothesis that the transition from Binding to Extrusion is an energy-requiring step in the transport process.

1.2. Simulation Design

The wild-type structure of MtrD was prepared for MD simulations by repairing missing residues with Modeller (residues 1, 494-507, 671-672, 1041-1056 of the 4MT1 structure) [20, 31]. The repaired monomer was assembled into a complete homotrimer using the crystallographic coordinates embedded in the PDB file. The structure was briefly minimized to allow the modeled loops to relax prior to simulations. We note that these loops are not located in regions that contact transport substrates or interface with neighboring MtrD monomers. Additionally, principal component analyses revealed that the Modeller-repaired loops were quite stable in simulations.
Once the protein was prepared for simulations, a model of the gram negative bacterial inner membrane was assembled using the CHARMM-GUI membrane builder [32]. The ratios of lipid components were based upon those in [33], whose in vitro model of the gram negative inner membrane included 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (POPE), 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (POPG) and 1,1′,2,2′-tetra-(9Z-octadecenoyl) cardiolipin (CL). The study used lipids purchased from Avanti Polar Lipids in the following ratio: 70% POPE : 20% POPG : 10% CL [33, 34]. Therefore, the structures of POPE, POPG, and cardiolipin from Avanti Polar Lipids were used to guide the selection of membrane components in the CHARMM-GUI membrane builder. Details of our model membrane that was used in subsequent MD simulations with NAMD are described in Table 1.2.1.

The choice to include both POPG and cardiolipin arose from the desire to mimic the native environment of MtrD as faithfully as possible – within current limitations. Simulations with a homogeneous POPE membrane would have run much faster than those with a heterogeneous membrane. However, a recent study of the MtrD homologue AcrB supports the hypothesis that the “membrane matters”, even in MD simulations. This study by Du et al. revealed that both POPG and cardiolipin preferentially associate with the transmembrane domains of the AcrB during MD simulations, even if the model membrane only contains a small fraction of POPG and/or cardiolipin at the start of the simulation [35]. Furthermore, cardiolipin was found to modulate the conformational dynamics of AcrB itself, including the transport of the AcrB substrate chloramphenicol. These data bolster our decision to sacrifice simulation speed to use a more native-like membrane environment, and suggest that more MD studies of lipid-RND transporter dynamics are warranted.
With the above considerations, the membrane was assembled, solvated, and neutralized with 0.15 mol/L NaCl. Prior to its use in simulations with MtrD, the model membrane was equilibrated using the CHARMM-GUI-generated inputs for NAMD. The MtrD homotrimer was then embedded into the heterogeneous membrane using coordinates from the OPM ( Orientations of Proteins in Membranes) Michigan database [36]. Notably, the position of MtrD within the lipid bilayer was later confirmed when the structure of MtrD\textsubscript{CR103} was solved in lipid nanodiscs [17]. The membrane-protein system was subsequently solvated with TIP3P water and neutralized with a final NaCl concentration of 0.15 mol/L, and this complete system was used for all MD simulations of MtrD with the NAMD software.

<table>
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<th>Lower leaflet number</th>
<th>Total %</th>
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Table 1.2.1. **Membrane Lipids used for MD Simulations with NAMD.** Lipids were chosen and assembled using the CHARMM-GUI membrane builder. Lipids were added by specifying the exact number of lipids per leaflet. CHARMM-GUI offers two cardiolipin (CL) species that correspond with the length of those used in [33]; consequently, a mix of the two was used. The lipid structures are provided by the CHARMM-GUI website (http://www.charmm-gui.org/?doc=input/membrane.bilayer).
1.3, Results

Using TMD simulations directed by known conformations of MtrD homologues, we simulated a putative catalytic transport cycle of MtrD by applying small forces to the α-carbons of the protein backbone (Figure 1.1.1E,F, Figure 1.1.2). As ligands for our MD simulations, we chose the MtrD substrate, azithromycin (AZY), and the MtrD non-substrate, streptomycin (SRY) [7, 9].

1.3.1. Generating Starting Sites for Ligands in TMD Simulations

The periplasmic cleft of MtrD is divided into the Access Pocket and the Deep Pocket, which are composed of the PC1/PC2 domains and the PN1/PN2 domains, respectively (Figure 1.3.1A). Within the Access and Deep Pockets are the proximal and distal binding sites of MtrD (Figure 1.3.1A). The proximal binding site of MtrD has been proposed to consist of charged, polar and hydrophobic residues that are primarily contributed by PC1, PC2 and PN2, (Figure 1.3.1A,B); in contrast, the distal site is thought to be composed of mostly hydrophobic residues from PC1, PN2, and residue 610 of the G-Loop (Figure 1.3.1A,C) [7, 17, 20]. Structures of the MtrD homolog AcrB suggest that large substrates bind first to the proximal site in the Access Pocket, and then subsequently pass the G-Loop into the Deep Pocket (Figure 1.1.1C,E,F) [23, 30]. The PC1/PN2 cleft forms a narrow alternative entry site for small, hydrophobic molecules (Figure 1.3.1A), but due to their large sizes, both SRY and AZY (~581 Daltons, ~749 Daltons, respectively) cannot pass through this narrow point of entry, and must enter through the Access Pocket.
Figure 1.3.1. The Drug Binding and Ligand Starting Sites of the Periplasmic Cleft.
A) The periplasmic cleft shown from the top down. The Deep Pocket (green) and Access Pocket (yellow) are outlined in dashed lines. The putative proximal and distal binding sites are shaded orange and green, respectively. The two possible entry pathways are numbered 1) the main entry path, and the 2) the alternate entry site that is only available to small substrates. B) The putative proximal binding site viewed from the side. Residues that may be important for substrate selectivity are shown in yellow sticks; residues of the proximal site that are conserved in AcrB are colored orange; the G-Loop is presented in magenta [17]. C) The putative distal binding site; residues that may be important for substrate selectivity are yellow sticks, proximal site residues that are conserved in AcrB are orange, distal site residues that are conserved in AcrB are dark green; G-Loop is magenta [17]. D) Ligand starting sites for TMD simulations, AZY in lime green, SRY in maroon.

Structures of AcrB indicate that the macrolide erythromycin binds at the entrance of the periplasmic cleft [30]. Several residues of the MtrD proximal binding site (residues in orange) are conserved in AcrB, and two of these – K714 and K823 – are thought to contribute to macrolide recognition (Figure 1.3.1B,C) [17]. Due to a dearth of structural data, the exact binding location of AZY or SRY within the proximal binding site of MtrD remains unclear. Since AZY is a macrolide and quite similar to erythromycin (Figure 1.3.2), it is reasonable to assume that AZY might bind in the homologous region of the proximal site of MtrD. However,
as we discuss later in this work, the Access Pocket – and the proximal site within – can accommodate multiple binding modes of azithromycin, despite its significant size.

Figure 1.3.2. **Structures of Azithromycin, Streptomycin, and Erythromycin.** Panels A–C show the molecular structure, protonation sites, and relative pKₐs of A) azithromycin (AZY), B) streptomycin (SRY), and C) erythromycin [37, 38]. Azithromycin and Erythromycin are both macrolides, and are also substrates of MtrD [7, 17]. Structures were sourced from the PubChem database, and pKₐs were sourced from [37, 38].

To generate reasonable starting positions of AZY and SRY for TMD simulations, we independently docked each ligand to the periplasmic cleft of MtrD using *Autodock Vina* [39]. Docking search areas sampled the entire periplasmic cleft (Figure 1.3.3). The resultant docking sites of SRY and AZY are shown in Fig S3. Notably, the predicted docking poses for each ligand clustered near the G-Loop, with outliers at the entrance or exit of the cleft (Figure 1.3.3). These docking studies suggest that the Access conformation of the periplasmic can accommodate multiple binding modes of AZY or SRY.
Figure 1.3.3. **Docking of AZY and SRY to the Periplasmic Cleft of MtrD.**
Docking experiments were performed with Autodock Vina and an exhaustiveness of 128 (default is 8). (A-B) The Access Pocket and Deep Pocket docking boxes (26 x 30 x 28). (C-D) The Left Pocket and Right Pocket docking boxes (36 x 30 x 30). Panels (E-J) show the periplasmic cleft of MtrD viewed from the top (i.e. periplasm) down, with helices of the Access Pocket colored yellow, and helices of the Deep Pocket colored green. Docking poses of SRY and AZY are shown in stick representation. (E) Docking results of AZY overlaid from all four dock boxes. (F) The most occupied cluster for AZY, with a panel showing the overlapping poses; this cluster was the basis for Site 1. (G) The second most occupied pose cluster of AZY; this cluster was the basis for Site 2. (H) Docking results of SRY overlaid from all four dock boxes. (I) The most occupied cluster for AZY; the basis for Site 1. (J) The second most occupied cluster; this cluster was the basis for Site 2.
We selected two poses to serve as ligand “start sites” for TMD simulations. In Site 1, SRY or AZY associates with the G-Loop and interacts with residues of the proximal binding site near the middle of the periplasmic cleft (Figure 1.3.1D, left panel). In Site 2, SRY or AZY is bound near the entrance of the cleft, and interacts with the foremost residues of the proximal binding site (Figure 1.3.1D, right panel).

1.3.2. Multiple Protonation States of AZY are Included in TMD Simulations

The broad substrate profile of RND transporters contributes significantly to their effectiveness as mechanisms of MDR [13]. One question to be asked, therefore, is whether the substrate profile of MtrD extends to different protonation states of the same substrate. At physiological pH ranges (between 7 and 7.5), three potential protonation states of AZY are possible: an uncharged form ($\text{AZY}_{\text{neu}}$); a singly protonated, positively charged form ($\text{AZY}_{\text{h1}}$); and a doubly protonated, positively charged form ($\text{AZY}_{\text{h2}}$) (Figure 1.3.2) [37, 40]. Conversely, only one protonation species of SRY is likely to occur at physiological pH ranges (Figure 1.3.2).

To investigate each form of AZY that might interact with MtrD, we included each possible protonation states of AZY our TMD simulations. Additionally, we note that the ionizable residues of MtrD are protonated (by default) according to a physiological pH of 7.4 in our MD simulations with both AMBER and NAMD. Histidines, which ionize in free solvent at a physiological pH of 7.4, default to protonation of the $\epsilon$ nitrogen in both NAMD and AMBER simulations [26, 41].

1.3.3. Defining Ligand “Transport” in TMD Simulations

Large substrates are thought to travel from the Access Pocket to the Deep Pocket during transport [6]. Thus, it was of interest to quantify this movement, if any, during TMD simulations. Two metrics were used for quantification: first, we calculated the root mean squared deviation
(RMSD) of the ligand from its starting position over time. In our system, the protein is oriented such that substrates, when moving in the transport direction (away from the Access Pocket), move on a diagonal in the X-Y plane. Thus, RMSD$_{\text{ligand}}$ provides a measure of ligand movement without necessitating quantification along two axes. However, RMSD calculations do not differentiate translational movement from rotational movements with little or no translational displacement. As a second measure of movement, we calculated the distance between the ligand’s center of mass (Ligand$_{\text{COM}}$) and specific residues of the periplasmic cleft interior. We chose the $\alpha$-carbons of R714 and T128 as our reference points (Figure 1.3.1A). Each was chosen based upon its position – and lack of significant movement – within the cleft. R714 and T128 primarily move on an axis perpendicular to the transport direction. If a ligand is transported, the Ligand$_{\text{COM}}$:R714$_{\alpha}$-carbon distance should increase, and the Ligand$_{\text{COM}}$:T128$_{\alpha}$-carbon distance should decrease (Figure 1.3.1A). We defined a ligand as “transported by MtrD” if, by the end-of-simulation, each of the following conditions was met:

1) the distance between the Ligand$_{\text{COM}}$ and R714$_{\alpha}$-carbon was greater than or equal to 18 Å;
2) the distance between the Ligand$_{\text{COM}}$ and T128$_{\alpha}$-carbon was less than or equal to 15 Å;
3) the ligand RMSD was at least 8.5 Å.

1.3.4. Azithromycin is Transported by MtrD in TMD Simulations

Using conformations of MtrD homologues as targets to model a putative transport cycle (Figure 1.1.2), we performed 20 independent TMD simulations per protonation state of AZY at both Site 1 and Site 2 ($n = 20$ TMD simulations per ligand, per start site). Based upon the previously defined distance cutoffs between Ligand(s)$_{\text{COM}}$ and R714$_{\alpha}$-carbon or T128$_{\alpha}$-carbon, simulation outcomes were divided into two clusters: Transported (Figure 1.3.4) and Non-Transported trajectories (Figure 1.3.5).
For each protonation state of AZY, we observed the following frequencies of transport: AZY_{neu} was transported in 12/20 simulations; AZY_{h1} was transported in 3/20 simulations; AZY_{h2} was transported in 6/20 simulations. Furthermore, within the “Non-Transport” cluster of AZY trajectories, two possible outcomes were observed: either 1) AZY remained in the same relative position straddling the G-Loop, or 2) AZY traveled into the Deep Pocket but remained closely associated with the G-Loop. Simulation endpoints of both Transport and Non-Transport trajectories are shown in Figure 1.3.7.
Figure 1.3.4 Transport of Azithromycin by MtrD in TMD Simulations.

A-C) show the RMSD$_{AZY}$ over time and the distance between COM$_{AZY}$, T128$_{a}$-carbon or R714$_{a}$-carbon. D) is a representative trajectory that shows transport of AZY (lime green) through the periplasmic cleft. A transparent blue overlay shows the approximate size of the Access Pocket. The G-Loop is outlined in magenta. E-G) show the chemical nature of protein-AZY contacts over the course of the simulation. H-J) show the hydration of the SASA$_{AZY}$, or SASA$_{distal}$hydrophobic, over the course of the simulation (see Methods). Data represent the mean ± one standard deviation in shading. Dashed grey lines indicate when MtrD reaches a structural checkpoint. MtrD begins in the ‘A’ (Access) conformation, and transitions to the following: ‘B’ (Binding), ‘E’ (Extrusion), and ‘I’ (Intermediate); timepoints between the dashed lines indicate structural transitions between two states.
Figure 1.3.5 Results of the “Non-Transport” Cluster of AZY at Site 1.
A-C) show the RMSD_{AZY} over time and the distance between COM_{AZY}, T128\alpha-carbon or R714\alpha-carbon. D) is a representative trajectory that shows transport of AZY (lime green) through the periplasmic cleft. A transparent blue overlay shows the approximate size of the Access Pocket. The G-Loop is outlined in magenta. E-G) show the chemical nature of protein-AZY contacts over the course of the simulation. H-J) show the hydration of the SASA_{AZY}, or SASA_{distal} hydrophobic, over the course of the simulation (see Methods). Data represent the mean ± one standard deviation in shading. Dashed grey lines indicate when MtrD reaches a structural checkpoint. MtrD begins in the ‘A’ (Access) conformation, and transitions to the following: ‘B’ (Binding), ‘E’ (Extrusion), and ‘I’ (Intermediate); timepoints between the dashed lines indicate structural transitions between two states.
1.3.5. Azithromycin Travels Farther When a Fourth “Intermediate” State is Simulated

In addition to the known Access, Binding and Extrusion conformations, a fourth potential ‘Resting’ structure was identified in CmeB from *Campylobacter jejuni*; CmeB shares a 38% sequence identity with MtrD (Figure 1.1.1E,F and Figure 1.1.2). The Resting conformation was classified by 1) the closure of the Access Pocket to the periplasm, and 2) the orientation of the transmembrane helices, which mimics that of the Extrusion conformation [21]. Notably, Su et al. found that the putative Resting conformation occurred in the absence of a transport ligand and was most stable in the absence of a proton gradient [21]. Considering these data, Su et al. postulated that ‘Resting’ could be a low-energy resting conformation. It should be noted, however, that a proton gradient across the plasma membrane is nearly always present in actively reproducing gram-negative bacteria [42].

Additionally, we found that the inclusion of the Resting conformation as the last step of the TMD sequence resulted in the additional movement of AZY in the transport direction (Figure 1.3.4A-C, “E → I”). Consequently, we hypothesize that the Resting structure could serve as an intermediate between Extrusion and Access and may even serve to position particularly bulky substrates for dissociation. Thus, we refer to this conformation as the “Intermediate” structure, as it may be an intermediate conformation that occurs during the transition from Extrusion back to Access (Figure 1.3.4, abbreviated as “I”).

An additional consideration for our TMD simulations is the relationship between the conformational changes of the periplasmic cleft and ligand dissociation into the funnel domain. It is unclear whether (1) peristaltic motions of the cleft squeeze the substrate into the funnel domain, or (2) the monomer adopts the Extrusion conformation and then “waits” for the ligand to disassociate. Since forces were not applied to ligands in our simulations, and the exact timing of
substrate release is unknown, we were uncertain if we would observe ligand dissociation in our TMD simulations. To prepare for the possibility, we modeled a full putative catalytic transport cycle, including the fourth putative Intermediate as the last step (S1 Fig).

We did not observe the movement of AZY in any protonation state into the funnel domain in our MD simulations. This is unsurprising, since an earlier computational study of the AcrB substrate Doxorubicin relied upon steered MD forces to pull Doxorubicin from the distal binding site, thereby forcing dissociation [43]. However, with the inclusion of the putative Intermediate state in our target sequence, we observed that AZY traveled farther through the periplasmic cleft (2.5 - 3 Å increase in distance traveled) and was in a more favorable position for release/dissociation events (Figure 1.3.4D, ‘Intermediate’).

1.3.6. Hydration of AZY Mediates Transport

Large substrates, particularly macrolides like AZY, are thought to bind at the distal site when the monomer is in the Binding state, and protein-macrolide contacts are thought to be dominated by hydrophobic interactions (Figure 1.3.1C) [17, 23, 44]. The cryo-EM structure of MtrD<sub>CR103</sub> shows erythromycin bound at the distal site in the PC1/PN2 cleft. In this structure (PDB ID 6VKT, chain B), we observed that 71% of protein-erythromycin contacts were contributed by hydrophobic residues, and 21% and 7% by polar or positively charged residues, respectively [17]. Of these contacts, we calculated that 43% were contributed by PN2, 43% by PC1, and 14% by the G-Loop. Also, we found that the residues involved are conserved between wild-type MtrD and MtrD<sub>CR103</sub> [17, 20]. Therefore, when MtrD was approaching the Binding conformation in our simulations, we expected that AZY would interact closely with the distal binding site – primarily with PN2, PC1 and the G-Loop. When AZY was transported, we indeed found that the G-Loop accounted for a significant share (21-30%) of protein-AZY contacts in the
Binding conformation (Table 1.3.1). However, we observed that most of the protein-AZY contacts (39-43% at Binding) were contributed by PN1, whose role in substrate extrusion has not been previously studied, and that PN2 and PC1 contributed only a small fraction of contacts, from 6 to 15% and 0.6 to 7%, respectively. (Table 1.3.1).

<table>
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<th>G-Loop</th>
<th>PC1</th>
<th>PC2</th>
<th>PN1</th>
<th>PN2</th>
<th>F-Loop</th>
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</table>

Table 1.3.1 MtrD-AZY Contacts in the Transport Cluster from Site 1.
Contacts are defined as residues whose α-carbon is within 4 Å of AZY at each timepoint of the simulation. Individual domains of the periplasmic cleft are as defined in Figure 1.3.1, and the % protein contacts for the Access Pocket (PC1 & PC2) and Deep Pocket (PN1 & PN2) have also been calculated from the mean contact value. Contact analysis was performed for trajectories in the “Transport” cluster using VMD and analyzed with scripts in Tcl and python. A: Access, B: Binding, E: Extrusion, I: Intermediate.
Based upon the structure of erythromycin-bound MtrD_Cr103, we also expected that the majority (~70%) of protein-AZY contacts at Binding would be contributed by hydrophobic residues; instead, we found that polar and hydrophobic residues each contributed ~40% of contacts (except for AZY_h1, where polar contacts outnumbered hydrophobic contacts) (Figure 1.3.4E-G). At Binding, this difference from the expected value can be attributed to the share of contacts contributed by PN1, which are mostly polar in nature, and the lack of (expected) contacts with the hydrophobic patch formed by PC1 and PN2.

Here it is helpful to note that, for the purposes of this work, “contacts” are defined as residues of MtrD within 4 Å of AZY at a particular timepoint of the simulation. The diameter of
a water molecule is ~2.8 Å [45]. Thus, it is possible for a hydrophobic residue to be counted as a
“contact” with AZY by our analysis scripts, but to also be separated from AZY by a layer of
water, which would prevent a true hydrophobic interaction from occurring via the occlusion of
water molecules between AZY and the residue of interest. To further elucidate the nature of
protein-AZY contacts, we calculated the percent hydration of the solvent accessible surface area
(SASA) of AZY and of the hydrophobic residues of the distal site – SASA_{distal,hydrophobic} – over
the course of the TMD simulations (Figure 1.3.4H-J).

When AZY was transported, we observed that the SASA_{AZY} was \geq 45\% hydrated
throughout transport. Notably, the SASA_{distal,hydrophobic} was \geq 60\% hydrated throughout the entire
simulation as well. When MtrD was in the Binding conformation, the point at which AZY was
expected to associate with these hydrophobic residues, the SASA_{distal,hydrophobic} increases to 80-
90\% (Figure 1.3.4H-J). Furthermore, the ligand is associating primarily with PC2/PN1 and the
G-Loop, not with PC1/PN2 (Table 1.3.1). These data suggest that significant hydrophobic
interactions between the distal site and AZY are unlikely to occur at the Binding stage of our
TMD simulations. However, the hydration of SASA_{AZY} and SASA_{distal} exhibited similar trends
between the Transport and Non-Transport clusters (Figure 1.3.4, Figure 1.3.5). Furthermore, the
majority of protein-AZY contacts were contributed by PN1, which has not previously been
suggested to contribute to substrate recognition or extrusion (Table 1.3.1). The second largest
share of contacts was contributed by hydrophobic residues of the G-Loop, which is thought to
filter and orient potential substrates throughout the transport process (Table 1.3.1).

While our data support the hypothesis that water mediates substrate movement through
the periplasmic cleft [43], our data also suggest that something other than water is causing AZY
to travel farther in the Transport cluster. As mentioned previously, the closing of the periplasmic
cleft during Extrusion (or the putative Intermediate state) causes AZY to move towards the funnel domain. This conformational transition bars access to the entrance of the periplasmic cleft and also significantly restricts the volume of the cleft itself. This is particularly apparent in the case of the Intermediate (aka “Resting”) structure, in which the cleft is almost entirely closed except for an area quite close to the exit [21]. In light of these data, we hypothesize that the transport of AZY by MtrD is mediated by 1) protein-ligand interactions, 2) gated access to specific areas of the periplasmic cleft, 3) a peristaltic-like squeezing caused by the cycle of conformational changes, and 4) hydration of the substrate.

1.3.7. Streptomycin was not Transported by MtrD in TMD Simulations

In TMD simulations with SRY from Site 1, using the same simulation routines and transport criteria as for AZY, SRY was classified as ‘not transported’ in 20 out of 20 trajectories (Figure 1.3.6). The mean RMSD$_{SRY}$ was 6.2 ± 1.7 Å (Figure 1.3.6A). The starting position of SRY at Site 1 is shown in Figure 1.3.6B, and the ending positions of SRY in all trajectories, as well as representative TMD simulations, are shown in Figure 1.3.6C. As shown in Figure 1.3.6C, SRY remains closely associated with the G-Loop, particularly F612 and S613, throughout the TMD simulations. While hydrogen bonding between SRY and S91 is predicted to occur in our post-simulation analyses, over 75% of the available SASA of SRY, including the guanidinium group that contacts S91, was hydrated throughout all of our TMD simulations (Figure 1.3.6D).

Furthermore, we found that polar contacts from PN1 and PC2 contribute the greatest share of MtrD-SRY contacts throughout the simulated transport cycle, followed by hydrophobic interactions with the G-Loop (Figure 1.3.6C,E and Table 1.3.3). SRY was not observed to interact significantly with the PC1 or PN2 domains (Table 1.3.3). We also observed that SRY displayed significantly more conformational flexibility than AZY. Our results indicate that SRY
does not exhibit transport behavior in our simulations, even if placed directly in the proximal binding site of MtrD (Site 1, Figure 1.3.1).

Figure 1.3.6 Interactions of Streptomycin with the Periplasmic Cleft at Site 1. Panel A) shows the RMSD_{SRY} from its starting position over time, and the distance between the center of mass of SRY and the α-carbons of T128 or R714 in the periplasmic cleft. B) shows SRY at Site 1 in the Access Pocket at the beginning of the TMD Simulations. C) shows all endpoints of SRY superimposed; one ending position of SRY, the position closest to fulfilling the criteria for “Transport”, is shown in opaque licorice representation; the other “non-transport” ending positions are in semi-transparent spacefill representation. A green dashed line marks the ending position of AZY in a transport trajectory as a reference. D) shows the percent (%) hydration of the SASA_{SRY} over the course of the simulation. E) shows the nature of protein-SRY contacts formed throughout the simulation. In B) and C), labeled residues in orange sticks interact with SRY in at least 80% of the TMD simulations with the G-Loop in magenta.
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Table 1.3.3. **MtrD-SRY Contacts Contributed by Domain of the Periplasmic Cleft.**

Contacts are defined as residues whose α-carbon is within 4 Å of SRY at each timepoint of the simulation. Individual domains of the periplasmic cleft are as defined in Figure 1.3.1, and the % protein contacts for the Access Pocket (PC1 and PC2) and Deep Pocket (PN1 and PN2) have also been calculated from the mean contact value. Contact analysis was performed for trajectories in the “Transport” cluster using VMD and analyzed with scripts in Tcl and python. A: Access, B: Binding, E: Extrusion, I: Intermediate.

1.3.8. **Azithromycin Exhibits Variable Behavior at the Periplasmic Cleft Entrance**

Although AZY is a known substrate of MtrD, due to its large size and the timescale of our TMD simulations, we did not expect to see AZY diffuse from the periplasmic cleft entrance towards the G-Loop. Indeed, if the initial process of G-Loop association is a passive diffusional process, we would not expect to observe AZY interaction with the G-loop when simulated from Site 2 (Figure 1.3.1). Consistent with these expectations, when AZY was placed at the very entrance of the periplasmic cleft, it largely remained between the PC1 and PC2 domains for the duration of the subsequent TMD simulations. In 2/20 simulations, AZY$_{neu}$ slipped from the periplasmic cleft into the periplasmic space but remained closely associated with its outer surfaces. This occurred in 1/20 simulations for AZY$_{h1}$, and 1/20 for AZY$_{h2}$. Despite its rarity, this behavior is unsurprising, given the fact that the cleft closed before AZY could associate with the G-Loop. In combination with our Site 1 data, these simulations suggest that the initial process of substrate capture – and movement towards the G-Loop – is likely a slow process. The full results of TMD simulations with AZY at Site 2 are shown in Figure 1.3.7.
Figure 1.3.7 TMD Simulation Starting and Ending Points.
All panels show the periplasmic cleft of MtrD viewed from the top down. The G-Loop is outlined in magenta, K823 is orange licorice representation, SRY and AZY are in spacefill representation, AZY is green, and SRY is maroon. A) and B) show the starting position of SRY at A) Site 1 and B) Site 2. The left image shows SRY in its starting position. The right image shows all of the ending positions of SRY superimposed. C) shows the results of simulations with AZY from Site 1; C1) shows the ending position of AZY in all ‘Non-Transport’ trajectories, and C2) shows the ending position of AZY in all ‘Transport’ trajectories. D) shows the results of simulations with AZY from Site 2; D1) shows the ending position of AZY divided by protonation state.
1.3.9. **Azithromycin Interacts with the Unique Portion of TM9 in TMD Simulations**

Although the diffusion of AZY towards the G-Loop was not observed in our simulations from Site 2, we unexpectedly observed close interactions between AZY$_{h1}$ and the TM9 linker, a region which is thought to be unique to MtrD among RND transporters [20]. These interactions were also observed with AZY$_{h2}$, but not with AZY$_{neu}$. The uppermost portion of TM9 and its linker (residues 917-927) projects away from the main “body” of the homotrimer and into the periplasm [20].

It has been suggested that RND transporters can capture substrates from the inner membrane-periplasm interface or from the periplasm [2]. Both AZY$_{h1}$ and AZY$_{h2}$ are weakly positively charged, and since the inner membranes of gram negative bacteria contain negatively-charged POPG lipids, it is possible that these charged AZY species would associate favorably with the periplasmic leaflet of the inner membrane prior to capture by MtrD [46]. TM9 projects out and away from the entrance to the periplasmic cleft, thus a bulky substrate would presumably contact TM9 prior to entering the periplasmic cleft. Notably, bulky substrates cannot enter directly through the uppermost regions of the cleft entrance due to the presence of the membrane fusion protein [47]. Therefore, it is possible that the uppermost region of TM9, which is composed of mostly polar or charged residues, could assist in capture of charged, amphiphilic substrates of MtrD. The role of this unique region of TM9 warrants further study.

1.3.10. **Streptomycin is Rejected from the Entrance of the Periplasmic Cleft**

Since SRY is not a known substrate of MtrD, we acknowledge that the positioning of SRY within the periplasmic cleft at Site 1 (Figure 1.3.1D) may be unlikely to occur naturally [9]. To test this assumption, we examined our results from Site 2 to see if SRY could diffuse past the ‘selectivity filter’ formed by residues at the cleft entrance (Figure 1.3.1C-D).
In contrast to the behavior of AZY in any simulation performed by us, we observed SRY move ‘backwards’, i.e. away from the interior of the periplasmic cleft. In 20 out of 20 subsequent TMD simulations, SRY dissociated from the interior of the periplasmic cleft and moved further into the solvent but remained associated with the outer surfaces of the cleft (Figure 1.3.7). At Site 2, SRY is in close contact with two positively charged residues – R714 and K823 – both of which are thought to be important for macrolide recognition [17]. Since SRY is strongly positively charged, it is possible that a combination of 1) repulsive interactions with R714 and K823, and 2) unfavorable contacts with the mostly hydrophobic PC1 domain, contributed to the passive rejection of SRY from the periplasmic cleft. Once SRY exited the periplasmic cleft, the most frequent residue contact occurred with D709 (>70% of the simulation time in 14/20 trajectories) on the outer surface of the cleft. The results of TMD simulations with SRY at Site 2 are shown in Figure 1.3.7.

1.3.11. The Molecular Landscape of the Periplasmic Cleft Changes Dynamically

To investigate how the dynamic molecular landscape of the periplasmic cleft might facilitate substrate capture and diffusion, we performed analyses of the Molecular Lipophilicity Potential (MLP) and the Electrostatic Potential (EP) isosurfaces of the periplasmic cleft.

1.3.12. Molecular Lipophilicity Potential (MLP) of the Periplasmic Cleft

MLP describes the distribution of lipophilicity (i.e., affinity for polar or non-polar solvents) across a molecular surface; MLP is calculated by summing the lipophilic contributions of molecular fragments upon the chosen point in space [48]. While a positive (+) MLP value indicates a lipophilic isosurface (Figure 1.3.8, gold regions), a negative (-) MLP indicates a hydrophilic isosurface (Figure 1.3.8, teal regions). Since MLP describes lipophilicity in three dimensions, the MLP of a molecular surface is sensitive to changes in the structure or
conformation of that surface. It is thought that ligand-receptor interactions may involve the interaction of complementary MLP patches (i.e. hydrophobic-hydrophobic), whereas mosaic MLP isosurfaces may contribute to weak, dispersive binding forces that might facilitate movement of a substrate across a surface [19, 24]. The first image of Figure 1.3.8A, which shows one half of the cleft in the Access conformation, illustrates a mosaic-like MLP surface composed of a patchwork of MLP-neutral or less-lipophilic surfaces. The second image of Figure 1.3.8A, showing the other half of the periplasmic cleft, shows an example of a large patch of surface area with a strongly lipophilic MLP.
Figure 1.3.8 The MLP Surfaces of the Periplasmic Cleft Change Dynamically. Panels show the Molecular Lipophilicity Potential (MLP) surfaces plotted on the molecular surface representation of the periplasmic cleft A) in the presence of AZYneu and B) in the absence of a Ligand. For visual aids, the channels formed by the periplasmic cleft at each conformation are approximated with dashed lines, the G-Loop is marked in magenta, and the direction of substrate travel is marked with a black arrow. MLP is colored teal to gold, from least lipophilic to most lipophilic. MLP was calculated using ChimeraX [49].

Through analysis of the AZY-bound or ligand-free monomer, we found that the available MLP isosurfaces of the periplasmic cleft change dynamically throughout the simulated transport
cycle. Figure 1.3.8A shows the MLP surfaces of AZY\textsubscript{neu}-bound MtrD, and Figure 1.3.8B shows the surfaces of ligand-free MtrD in the same conformation. In Access, the MLP of PC2 and PN1 was a mosaic of polar-neutral areas, whereas the MLP of PN2 and PC1 was much more lipophilic, particularly at the entrance of the periplasmic cleft and at the alternate entry site for small, hydrophobic substrates (Figure 1.3.8A-B, Access). Since MtrD is a member of the hydrophobic-amphiphile (HAE) family of RND transporters, it is unsurprising that these two areas of the periplasmic cleft – both of which are thought to function in the filtration and capture of substrates – consist almost entirely of lipophilic or weakly polar MLP isosurfaces.

In the Binding conformation, the differential distribution of MLP surfaces is still apparent, with PC2/PN1 creating a mosaic of hydrophilic and neutral surfaces, and PN2/PC1 creating a ‘lipophilic highway’ that, at first glance, seems to extend directly through the cleft (Figure 1.3.8A-B, Binding). However, access to the illusory ‘lipophilic highway’ is obstructed by the G-Loop, i.e. to associate with the lipophilic areas of the Deep Pocket, bulky substrates must slip past the G-Loop [7]. Indeed, our docking studies show that the G-Loop prevents AZY from maintaining consistent contact with the PC1/PN2 domains during the transition from the Access Pocket to the Deep Pocket. Instead, as shown in Figure 1.3.3, the predicted binding modes of AZY follow a pathway that curves to the left side of the G-Loop. Along this pathway, AZY primarily contacts PC2, PN1 and the G-Loop. PC2 and PN1 are composed of hydrophobic-neutral mosaic MLP isosurfaces (Figure 1.3.8, Binding), and as such, are particularly suited to facilitating the diffusion of an amphiphilic or hydrophobic substrate.

In the Extrusion conformation, we found that ligand access to PC1 and PC2 is barred by A) the closure of the cleft entrance and B) the G-Loop. These conformational changes prevent ‘backflow’ of the substrate into the Access Pocket. In the subsequent (putative) Intermediate
conformation, the volume of the Deep Pocket is further constricted, and the Access Pocket is still closed to the periplasm. In the Extrusion and Intermediate conformations, the majority of available MLP isosurfaces (to a bound ligand) were found to be hydrophilic or neutral, apart from the hydrophobic stopgap formed by the G-Loop (Figure 1.3.8A-B, Extrusion, Intermediate). These hydrophilic/neutral isosurfaces may serve to encourage substrates to leave the periplasmic cleft, as many MtrD substrates are hydrophobic or amphiphilic. Interestingly, analysis of the AZY-bound monomer reveals that the presence of AZY only slightly changed the MLP signatures of the periplasmic cleft (Figure 1.3.8B).

Therefore, through MLP analysis of the periplasmic cleft, we found that both substrate entry points contain significantly hydrophobic isosurfaces, and the only known exit consists of primarily hydrophilic-neutral isosurfaces. Therefore, it appeared that substrates first interacted with complementary isosurfaces, and were subsequently shuttled to uncomplimentary or neutral isosurfaces deeper within the periplasmic cleft. Once the substrate reached the Deep Pocket behind the G-Loop, the closure of the periplasmic cleft restricted access to hydrophobic areas within the cleft. It appears to us therefore, that throughout the substrate transport cycle, ligand access to hydrophobic areas of the cleft was alternately allowed and restricted.

1.3.13. Electrostatic Potential (EP) Isosurfaces of the Periplasmic Cleft

As with MLP, we found that the electrostatic potential (EP) isosurfaces of the periplasmic cleft change dynamically throughout the simulated transport process. However, in contrast to the results of the MLP analyses, we observed that the EP isosurfaces also changed significantly in the presence and absence of AZY. Figure 1.3.9A shows the EP of AZY

neutral-bound MtrD, and panel B shows the EP of ligand-free MtrD in the same conformation. In the absence of AZY, the periplasmic cleft contained mildly negative or neutral isosurfaces (Figure 1.3.9B). In the
presence of AZY, the cleft gained positive-neutral mosaic isosurfaces in the Access conformation and lost a significantly negative patch in the Binding conformation (Figure 1.3.9A).

Furthermore, in the Extrusion and Intermediate conformations, we found that K823 contributes a strongly positive region near the G-Loop (Figure 1.3.9A, Extrusion). Since forces were only applied to the α-carbons of MtrD, the side chains and ligand were allowed to move freely. Therefore, changes in the EP isosurfaces are due presumably to (1) changes in the orientation of side chains and (2) the presence of AZY within the periplasmic cleft. Since AZY$_{h1}$ and AZY$_{h2}$ are weakly positively charged, it is possible that the strongly positive patch near the G-Loop could repel AZY from the center of the cleft and towards the funnel domain of the transporter.
Figure 1.3.9 The EP Surfaces of the Cleft Change in the Presence of AZY.

Panels show the Electrostatic Potential surfaces plotted on the molecular surface representation of the periplasmic cleft A) in the presence of AZY_{neu} and B) in the absence of a Ligand. For visual aids, the channels formed by the periplasmic cleft at each conformation are approximated with dashed lines, the G-Loop is marked in magenta, and the direction of substrate travel is marked with a black arrow. EP is colored red to blue, from negative (-10 k_B T/e) to positive (+10 k_B T/e) potential, where k_B is the Boltzmann constant, T is the absolute temperature (310 K), and e is the electron charge. EP surfaces were calculated using the APBS online server and visualized with ChimeraX.
1.3.14. Does MtrC Play a Role in Substrate Recognition?

Regarding the interpretation of our MD simulations, a significant consideration is the lack of the membrane fusion protein MtrC. The MtrCDE complex assembles with a 3:6:3 stoichiometry; MtrC assembles as a hexamer, while MtrD and MtrE both assemble as trimers [19]. Since the structure of MtrC remains unsolved, we built a homology model using fully assembled AcrA from the AcrAB-TolC complex [47]. AcrA shares a 44.6% sequence identity with MtrC (PDB ID 5NG5) [47]. We then built a full model of MtrCDE using the structures of MtrD (4MT1), MtrE (4MTO), and the model of MtrC [20, 50]. To assemble MtrCDE, the fully assembled AcrAB-TolC structure (5NG5) was used as a template, since this homologous system also assembles with a 3:6:3 stoichiometry. The resultant model of MtrCDE is shown in Figure 1.3.10.
Figure 1.3.10. **Homology Model of Fully Assembled MtrCDE.**
A) shows our fully assembled homology model of MtrCDE; the model was assembled using the 5NG5 structure of AcrAB-TolC as a structural template (see Methods), the Wild Type structure of Apo MtrD (4MT1), and the structure of MtrE in the open conformation (4MTO) [13, 35, 38]. B) The MLP (molecular lipophilicity potential) of MtrCDE; the zoom-in panel shows the interface between MtrC (outlined in green) and MtrD (outlined in grey). C) The electrostatic potential (EP) surfaces of MtrC calculated by the PDB2PQR server. The MtrC-MtrD interface (that could potentially contact ligands) contains mostly neutral MLP and EP isosurfaces.

We found that two MtrC monomers significantly contact the periplasmic cleft of each MtrD monomer. As shown in Figure 1.3.10A, one monomer sits atop the PC1 and PC2 domains at the entrance of the periplasmic cleft of MtrD (1546.5 Å² total contact area). Another MtrC
monomer contacts the outer surfaces of the PN2 domain (909.3 Å² total contact area), which interfaces with the neighboring MtrD monomer. We note that the positioning of MtrC would prevent substrates from diffusing down through the top of the periplasmic cleft. Furthermore, due to its extensive contacts with the entrance of the periplasmic cleft, it is possible that MtrC plays a role in screening of substrates, particularly those of large molecular weight.

To investigate the role of MtrC in substrate recognition, our MtrC model was subjected to analyses of the MLP and EP isosurfaces as previously described. We found that the regions of MtrC that contact the periplasmic cleft, and particularly those that might contact bulky substrates, are a mosaic of weakly hydrophilic or neutral surfaces (Figure 1.3.10B-C). However, our docking studies exclusively identified docking poses for AZY in the lower regions of the periplasmic cleft (S3 Fig). This area is well separated from the MtrC contact regions, raising the question of whether a substrate would contact MtrC at all. Additionally, docking studies performed by Chitsaz et al. predicted binding poses for AZY almost exclusively within the Deep Pocket (i.e. behind the G-Loop) of the unequilibrated MtrD crystal structure (4MT1) [7]. Even though both docking studies of AZY were designed to fully sample the Access Pocket of MtrD, neither we nor Chitsaz et al. identified predicted binding poses of AZY within regions of the cleft that contact MtrC. Therefore, based upon the MtrCDE model and the best available evidence, we conclude that MtrC is unlikely to play a significant role in the identification and filtering of substrates.

1.3.15. AZY Diffuses Through the Periplasmic Cleft in a Long Timescale Simulation

Crystal structures of AcrB suggest that large substrates first associate with the G-Loop after entering the periplasmic cleft, and subsequently enter the Deep Pocket during the Access to Binding transition. MD studies of AcrB also suggest that the energy-requiring step in the
conformational sequence is the structural change between the Binding and Extrusion conformations [22]. In light of these results, we postulate that our AZY-bound MtrD system used in this long timescale simulation approximates a model of MtrD in the Access conformation with a substrate bound at the proximal site and closely associated with the G-Loop.

The structural transitions of the MtrD efflux cycle are slow and presumably powered by changes in the protonation state of the PRN [21]. To overcome these limitations, we used TMD in the previous studies to model a putative transport cycle without altering the protonation state of the transmembrane PRN. However, the applications of TMD can be limited due to the use of biasing forces and the lack of control over simulation timescales, depending upon the TMD implementation used. To address these limitations, we performed an unbiased simulation of AZY neu at Site 1 using the AMBER pmemd-cuda MD engine. This 1.5 µs simulation was performed at 310 K (Figure 1.3.11A), and external forces were not applied to any atom in this system. Furthermore, the protonation states of the PRN were unaltered.

In this long timescale, unbiased simulation, we observed AZY move past the G-Loop and into the Deep Pocket (Figure 1.3.11B, Table 1.3.4). Principle Component Analysis of the protein backbone reveals that significant fluctuations occurred in the PC2 domain, TM helix 8, and the docking domain of MtrD with MtrCE (Figure 1.3.11C). The docking domain is located at the very top of the MtrD transporter, and in the fully assembled complex, is completely covered by the MtrCE complex (Figure 1.3.10). Since MtrCE is absent from our simulations, fluctuations of the docking domain – which normally interfaces with the sizeable MtrCE complex – are unsurprising. However, structures of AcrB and the variant MtrD<sub>CR103</sub> (PDB IDs 5NC5, 6VKT) suggest that conformational changes in TM8 and the PC2 domain do occur, at some point, during the transition from Access to Binding [8, 35]. Specifically, 1) the upper portion of TM8 will
adopt a more ordered structure, eventually settling into a full alpha helix in the Extrusion conformation, and 2) the PC2 domain opens even wider (than in the Access conformation). Interestingly, we observed corresponding conformational shifts in both TM8 and PC2 in our simulation (Figure 13B,C). Specifically, TM8 shifted from a disordered loop to a more ordered structure (Figure 13A,B in blue), and PC2 opened wider once AZY had slipped into the Deep Pocket.
Figure 1.3.11 Dynamics of AZY and MtrD During a Long Timescale Simulation.

(A-B) Pre- and post-simulation snapshots of AZYneu and the periplasmic cleft, viewed from the top down, or viewed from the side (boxed view), showing AZY (green), the G-Loop (magenta), TM helix 8 (blue), and the PC2/PN1 cleft (outlined in red). Labeled residues interact with AZY for ~80% of the simulation. In (B), arrows show how the PC2/PN1 cleft shifts. (C) Results of Principle Component Analysis on the MtrD backbone. Principle components 1, 2 and 3 mapped onto the MtrD monomer. Red indicates areas of high structural fluctuation; blue indicates very low structural fluctuation. (D) The percentage of contacts between AZY and the periplasmic cleft that are charged, polar, or hydrophobic. (E) The hydration of the SASA of AZY or residues of the putative distal site throughout the simulation.
Table 1.3.4. **MtrD-AZY Contacts in a Long Timescale Simulation.**
Simulation contacts are defined as residues whose α-carbon is within 4 Å of AZY at each specified timepoint of the 1.5µs simulation with AMBER. Individual domains of the periplasmic cleft are as defined in Figure 3, and the % protein contacts for the Access Pocket (PC1 and PC2) and Deep Pocket (PN1 and PN2) are calculated from the mean contact value. Contact analysis was performed with scripts in Tcl and python.

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<td>40.5</td>
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<td>11.7</td>
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<tr>
<td>100%</td>
<td>11.2</td>
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<td>14.1</td>
<td>43.5</td>
<td>11.8</td>
<td>2.9</td>
<td>33.5</td>
<td>55.3</td>
</tr>
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</table>

While most MtrD-AZY contacts were hydrophobic or polar at the beginning of the simulation, the majority of contacts were contributed by hydrophobic residues by the end of the simulation (Figure 1.3.11D). The hydration of the $\text{SASA}_{\text{AZY}}$ decreases slightly overall as the simulation progresses; however, the $\text{SASA}_{\text{distal hydrophobic}}$ is $\geq 70\%$ hydrated for the majority of the simulation, indicating that significant hydrophobic interactions between these residues and AZY are unlikely to occur in our simulation Figure 1.3.11E).

Our results are in line with MD studies of AcrB that suggest that the transition from Binding to Extrusion is an energy-dependent step [15, 31]. Our results are also supported by the MD studies of Chitsaz et al, in which the MtrD substrate progesterone was observed to spontaneously move into the Access Pocket, past the G-Loop, and into the Deep Pocket [12]. Similar to our simulations, the movement of progesterone occurred in the absence of biasing.
forces, and without changes to the PRN. Interestingly, this study observed the movement of progesterone through the periplasmic cleft in ~40 ns; we postulate that the increased movement speed (relative to our simulation of AZY) could be due to the significant discrepancy in size between progesterone and AZY (~315 Da for progesterone vs. ~749 Da for AZY).

1.4. Discussion

In this study, we have simulated the transport of AZY by MtrD in biased Targeted Molecular Dynamics simulations, and in an unbiased, long-timescale NPT MD simulation. We also observed the passive rejection of SRY from the entrance of the periplasmic cleft, or alternatively the retention of SRY within the cleft during the putative transport cycle. We showed that the access to various isosurfaces of the periplasmic cleft changes dynamically, and that access is controlled in a way that might facilitate substrate discrimination, transport, and extrusion. In contrast to the interpretation of results from crystal structures AcrB and cryo-EM structures of MtrDCR103, AZY was not observed to interact with residues of the putative distal binding site in TMD simulations, but took an alternative transport pathway mediated by water, and by interactions with PN1 and the G-Loop. In our long timescale, unbiased MD simulation, we observed AZY diffuse into the Deep Pocket past the G-Loop, and we observed the AZY-bound monomer begin to undergo conformational changes that are associated with the transition from Access to Binding.

1.4.1. AZY Trajectories Diverge during the Transition from Access to Binding

With our TMD simulations, we were able to increase the sample size significantly to 20 simulations per ligand at each starting site. This larger sample size allowed us to observe variable behavior of AZY during the simulations. Through analysis of the specific domains contacted by AZY throughout the simulation, we found that the “Transport” trajectories diverged from the
“Non-Transport” trajectories during the transition from Access to Binding (Table 1.3.1, Table 1.3.2). Specifically, if AZY slipped past the G-Loop at some point during the transition from Access to Binding, then AZY was positioned fully behind the G-Loop, and subsequently squeezed towards the exit as the cleft entrance closes (during the transition from Binding to Extrusion). Notably, this movement of AZY into the Deep Pocket occurred during the conformational transition period, and thus before the periplasmic cleft had fully adopted the Binding conformation.

These data indicate that AZY can pass the G-Loop even if the cleft is not in its most open conformation. This correlates with the results of our long timescale simulation, in which AZY spontaneously slipped past the G-Loop and into the Deep Pocket, even though the transporter was not yet in the Binding conformation. This is also supported by the results of our docking experiments against apo-MtrD, which identified predicted binding positions of AZY that spanned the length of the periplasmic cleft (Figure 1.3.3). Lastly, this is supported by simulations of the MtrD homolog AcrB, in which steered MD techniques were used to pull the AcrB substrate chloramphenicol from the periplasm and into the Deep Pocket of the transporter in the Access conformation [35]. Even though chloramphenicol (323 Da) is half the molecular weight of AZY (749 Da), the periplasmic cleft, along with the inherent flexibility of the G-Loop, allows enough room for substrates to slip into the Deep Pocket – even though the periplasmic cleft is less ‘open’ in Access than in the Binding conformation.

1.4.2. Molecular Characteristics of the Cleft Facilitate Recognition and Diffusion of Substrates

Our MLP analyses revealed that the available MLP isosurfaces of the periplasmic cleft changes dynamically throughout the transport cycle. Areas that are responsible for substrate filtration and capture contain significantly hydrophobic isosurfaces, and in the case of the
alternate entryway site for small substrates, the passage is lined with bulky hydrophobic residues (Figure 1.3.1). These lipophilic isosurfaces may facilitate the substrate selectivity of MtrD, which is a member of the hydrophobic-amphiphile family of RND transporters, thereby allowing ligands with sufficient complementarity to enter the cleft and associate with the G-Loop. As shown with our simulations of SRY, bulky molecules with non-complementary characteristics are unable to pass reliably through this selectivity filter at the cleft entrance.

The MLP isosurfaces of the periplasmic cleft interior are mosaic-like; consequently, they are well-suited to allowing MtrD substrates to diffuse through the tunnel formed by the periplasmic cleft, as shown by Chitsaz et al. in their simulations of MtrD and its substrate progesterone [7]. Along with the presence of water [43], these mosaic-like surfaces of the interior [24] may prevent substrates from stabilizing inside the cleft as subsequent conformational motions squeeze them towards the funnel domain. Once substrates reach the Deep Pocket behind the G-Loop, access to lipophilic areas in the Access Pocket is restricted by closure of the PC1/PC2 domains. Except for the lipophilic stopgap formed by the G-Loop, the ligand-accessible isosurfaces are either hydrophilic-neutral or positive neutral (Figure 1.3.8, Figure 1.3.9, Panel A, ‘Extrusion’). The positive patch may repel charged substrates from the center of the cleft, thereby positioning substrates for release into the funnel domain.

Thus, the interior of the cleft contains 1) substrate-complementary surfaces to aid discrimination at the entrance, 2) mosaic-neutral surfaces to aid diffusion towards the funnel domain, and 3) mosaic-repulsive surfaces to aid diffusion and eventual extrusion of substrates. The correlated motions of the periplasmic cleft and of the G-Loop serve to alternately restrict and allow access to areas with specific MLP and EP signatures. While the MLP signatures of the
cleft do not appear to change significantly in the presence of AZY, the presence of AZY does appear to affect the EP isosurfaces of the cleft.

1.4.3. AZY did not Exit Fully into the Funnel Domain in Our Simulations

We did not observe the exit of AZY into the funnel domain (Figure 1.1.1) in any of our MD simulations. To our knowledge, no computational simulation of an RND transporter has modeled substrate release into the funnel/docking domain without the use of biasing forces upon the ligand or substrate itself [7, 51], which is consistent with the hypothesis that substrate release occurs on a longer timescale than is currently reasonable to simulate on most systems.

1.4.4. AZY did not interact with the ‘hydrophobic trap’ during the Binding conformation

Cryo-EM structures of MtrD_{CR103} show the macrolide erythromycin bound in the “hydrophobic trap” of the PC1/PN2 cleft (PDB 6VKT) [13, 16]. The ‘hydrophobic trap’ is a set of hydrophobic residues that forms the alternate entry site for small, hydrophobic substrates in the Deep Pocket (Figure 1.3.1C, green residues) [52]. Studies suggest that residues of the hydrophobic trap are quite important for the proper function of RND transporters [44, 52]; specifically regarding MtrD, mutations in this region (F136A, F176A, and F623C) correlate with a decrease in the MIC (Minimum Inhibitory Concentration) of the antibiotics rifampin (~823 Da), novobiocin (~613 Da), and oxacillin (~401 Da) [7]. Since none of these antibiotics are macrolides, the result of mutations in this region upon the MIC of macrolides warrants further study. Consequently, we expected that AZY would interact with the hydrophobic residues of the distal site when the transporter was in the Binding conformation.

Surprisingly, in our TMD simulations, we found that AZY took an alternate path through the Deep Pocket primarily involving PN1 and the G-Loop, and not PN2 and the hydrophobic trap as expected. In our long timescale simulation, we observed interactions between AZY and the
hydrophobic trap only at the end of the simulation, and these contacts were relegated to the periphery of the AZY molecule. At no point, in any of our simulations, was AZY bound in a conformation like that of erythromycin in the Deep Pocket of MtrD_{CR103}. Nevertheless, the role of the distal site (and the hydrophobic trap therein) in macrolide transport is of interest. One particularly important question that remains is whether the conformational change from Binding to Extrusion is enough to extrude a large substrate out of the hydrophobic trap and into the funnel domain?

To investigate this question, we modeled the structural transition from Binding to Extrusion using the erythromycin-bound monomer of MtrD_{CR103} (PDB ID 6VKT). We found that the putative distal site constricts significantly during this conformational change. We note that this constriction may be sufficient to squeeze a bulky substrate up into position for release into the funnel domain, but it is unclear whether it provides enough force to extrude the substrate. Therefore, it remains unclear whether the substrate exits into the funnel domain as the monomer transitions from Binding to Extrusion, or if the monomer adopts Extrusion and then waits for the substrate to disassociate. Our data suggest that substrate release into the funnel domain occurs slowly and after the monomer has reached Extrusion. Furthermore, our data indicate that the extrusion of bulky substrates may be aided by further constriction of the periplasmic cleft in the putative Intermediate (aka “Resting”) state.

1.4.5. The Selectivity Filter at the Cleft Entrance Facilitates the Passive Rejection of SRY

Our results demonstrate that SRY is unlikely to successfully enter the periplasmic cleft of MtrD, and thus will not be transported by MtrD. Since SRY is too large to pass through the alternate entry site behind the G-Loop, the only available point of entry is through the Access Pocket (AP) at the foremost region of the periplasmic cleft. Our simulations of SRY at the
periplasmic cleft entrance (Figure 1.3.1B, Site 2) support the conclusion that SRY is unlikely to successfully enter through the Access Pocket of MtrD. Nevertheless, while SRY was observed to diffuse out of the cleft and into the solvent, it did remain associated with the periplasmic-exposed surfaces of the AP (Figure 1.3.7). The outer surfaces of the AP possess negative patches, particularly near D709, that may attract SRY to these regions once it exits the cleft. Taken together, our results also indicate that SRY is an unlikely candidate for an MtrD inhibitor, since it is unable to enter the transporter successfully.

1.4.6. Mutations Alter the Molecular Landscape of the Periplasmic Cleft

Mutational studies have shown that a K823E mutation in the periplasmic cleft results in a fourfold increase in AZY resistance; consequently, K823 is thought the be important for macrolide recognition [7, 17]. We performed EP and MLP analysis of the MtrD periplasmic cleft with the K823E mutation and found that the cleft gains a strongly negative patch in the proximal binding site near the G-Loop and TM8. Since >90% of AZY are weakly positively charged at physiological pH, it is possible that this pocket would serve to further attract AZY into the periplasmic cleft after the initial association with the entrance of the cleft. Indeed, we observed close interactions between AZY and K823 throughout much of the long timescale simulation. Consequently, K823-macrolide interactions warrant further study.

Analysis of the K823E mutation raises an additional question: could MtrD_{K823E} transport SRY? When examining the MLP of the periplasmic cleft entrance, we found that PC1, which forms half of the entrance to the cleft, contains significant hydrophobic isosurfaces (Figure 1.3.8A-B). Positioned opposite to the hydrophobic surfaces of PC1 is the conserved, positively charged residue R714. Thus, for SRY to successfully enter the periplasmic cleft, it would need to pass the positively charged residue R714, as well as the significantly hydrophobic isosurfaces of
PC1, before reaching the K823E. As shown in Table 1.4.1, the topological polar surface area (TPSA) of SRY is nearly twice that of AZY$_{h2}$. As TPSA increases, hydrophilicity increases, and vice versa. Analysis of the Log $S$ (solubility in water) and the Log $P_{oct/wat}$ (octanol-water partitioning) reveals that SRY is significantly more hydrophilic than any of the three protonation states of AZY (S1 Table). Therefore, SRY is unlikely to interact favorably with the hydrophobic surfaces of PC1, or with R714 of PC2. Since AZY is significantly more lipophilic than SRY, AZY is more likely to interact favorably with these surfaces, and to successfully enter the periplasmic cleft. To our knowledge, the potential of MtrD$_{K823E}$ to transport SRY has not been assessed.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular Weight (g/mol)</th>
<th>TPSA, Å$^2$</th>
<th>Consensus Log $P_{oct/wat}$</th>
<th>Water Solubility (Log $S$)</th>
<th>H-bond acceptors</th>
<th>H-bond Donors</th>
</tr>
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<tr>
<td>SRY</td>
<td>548.6</td>
<td>344.23</td>
<td>-7.36</td>
<td>Highly soluble</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>AZY$_{neu}$</td>
<td>734.96</td>
<td>191.08</td>
<td>1.15</td>
<td>Poorly soluble</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>AZY$_{h1}$</td>
<td>735.97</td>
<td>191.28</td>
<td>0.13</td>
<td>Poorly soluble</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>AZY$_{h2}$</td>
<td>736.97</td>
<td>191.48</td>
<td>-0.89</td>
<td>Poorly soluble</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>ERY$_{neu}$</td>
<td>733.92</td>
<td>193.91</td>
<td>1.99</td>
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<td>5</td>
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<td>195.11</td>
<td>0.97</td>
<td>Soluble</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1.4.1 Predicted Molecular Characteristics of AZY, SRY, and Erythromycin. The physicochemical descriptors and properties of the three different protonation states of AZY, the single protonation state of SRY, and the two protonation states of erythromycin, were calculated using the SWISS-ADME server [53]. The consensus Log P is the average of the calculated LogP using five different methods. The Log $S$ indicates the predicted solubility in water. SWISS-ADME presents three different predictions of Log $S$ each using different methodology, with the potential classifications ‘highly soluble’, ‘soluble’, and ‘poorly soluble’. For each form of AZY, 2/3 methods predicted “poorly soluble”, the remaining method (SILICOS-IT) predicted “soluble”. For SRY, 2/3 methods predicted “highly soluble”, the remaining method (SILICOS-IT) predicted “soluble”. The Topological Polar Surface Area (TPSA) indicates the surface sum over all polar atoms in the respective molecule in Å$^2$; as TPSA increases, hydrophilicity increases.
1.4.7. Potential Effects of Different Forcefields

In this study, we demonstrated the transport of AZY, a known substrate of MtrD, in simulations with two types of force fields using two different MD engines – NAMD with CHARMM36 force fields, and AMBER pmemd-cuda with Amber force fields. This suggests that our results are not artifacts of the force fields employed.

1.4.8. Mutagenesis Experiments Support the Hypothesis of the “PN1 Route”

Co-crystallization of the related antibiotic erythromycin (ERY) with MtrD<sub>CR103</sub> shows ERY bound snugly at the distal site (Figure 1.4.1). Consequently, we expected AZY to associate with the distal site during our TMD simulations. We observed, however, that AZY deviated from the expected path through the periplasmic cleft and instead maintained contact primarily with the PN1 domain whilst moving in the transport direction (Table 1.3.1). Thus, the question arises – is the “PN1 Route” an artifact of the TMD simulations? We found that a recent study by Wadsworth et al. supports the existence of the “PN1 Route” for AZY (Figure 1.4.1A) [54].

Wadsworth et al. analyzed the sequences of MtrCDE and the MtrR promoter in clinically obtained specimens of <i>N. gonorrhoeae</i> from the Gonococcal Isolate Surveillance Project (GISP). Wadsworth et al. showed that mutations in the PC2 and PN1 domains of MtrD correlate with increased resistance to AZY. In a particularly elegant experiment, Wadsworth et al. transformed the <i>mtrD</i> locus in a non-AZY-resistant strain (<i>N. gonorrhoeae</i>, 28BI) with two portions of the <i>mtrD</i> locus from an AZY-resistant strain (<i>N. gonorrhoeae</i>, GCGS0276). These two regions, which correspond to the 5’ end and 3’ end of GCGS0276 <i>mtrD</i>, together produce the PN1 and PC2 domains of MtrD, and contain several mutations in regions of the periplasmic cleft that would contact AZY (20 mutations total) (Figure 1.4.1B). The substitution of these two domains from the AZY-resistant strain increased the (28BI, non-resistant strain) MIC for AZY fourfold.
Importantly, 28BI transformants involving other regions of MtrD from GCGS0276 did not raise the 28BI MIC of AZY, suggesting that PN1 and PC2 are indeed involved in the efflux of AZY [54]. These experiments strongly support the hypothesis that PN1, and by extension PC2, is involved in transport of AZY, and correlate well with the results of our TMD experiments.

Figure 1.4.1. **PC2 and PN1 Mutations Confer AZY Resistance.** Here we show two views of the periplasmic cleft. A) shows the periplasmic cleft viewed from the top down. A black arrow shows the expected path of AZY during transport, based upon co-crystallization of ERY at the Distal Site (green shaded area). An orange arrow shows the observed path of AZY during transport, dubbed the “PN1 Route”. B) shows the periplasmic cleft viewed from the side, with MtrD domains labeled. AZY is shown in yellow licorice representation. The G-Loop is in magenta. Residues in blue sticks are mutations found in the AZY-resistant GCGS0276 strain of *N. gonorrhoeae* [54]. Transforming the non-resistant 28BI strain with the PC2 and PC1 domains of GCGS0276 increased the AZY MIC of 28BI fourfold [54]. 28BI transformants with other domains of GCGS0276 did not exhibit an increase in the MIC of AZY.
1.5. Conclusion

On the basis of this work, we suggest the following transport mechanism for macrolides by MtrD (Figure 1.4.1 A).

1. A macrolide first associates with, and then passes, the selectivity filter at the entrance of the periplasmic cleft. This filter is formed by conserved residues and by isosurfaces that are complementary to the substrate profile of the transporter.

2. The macrolide diffuses slowly through the periplasmic cleft, contacting residues of the loosely defined proximal site, eventually associating with the G-Loop [17].

3. The macrolide slips past the G-Loop and into the Deep Pocket, still associating with the proximal site. Once the macrolide enters the Deep Pocket, the MtrD monomer undergoes the transition from Access to Binding, opening the periplasmic cleft even wider.

4. Once in the Deep Pocket, the macrolide may interact with the putative distal site, or with the PN1 domain. In both cases, it is still contacting the G-Loop. If the macrolide takes the ‘PN1 route’, it is technically still associating with residues of the proximal binding site.

5. Changes in the transmembrane Proton Relay Network then power the transition from Binding to Extrusion, in part through resultant vertical shearing motions in the transmembrane helices [55]. This restricts access to the periplasm and the Access Pocket, resulting in the extrusion of the substrate towards the funnel domain.
6. Once the monomer reaches the Extrusion conformation and the substrate is positioned for disassociation into the funnel domain, the monomer waits for the relatively slow process of substrate release.

7. During this waiting period, the monomer could relax and pass through the putative Intermediate conformation, which may serve to further move bulky substrates out of the transport channel.

In conclusion, our data suggest that substrate transport through the periplasmic cleft of MtrD depends upon a combination of diffusion, gated access to areas with variable charge and lipophilicity isosurfaces, and conformational changes of the surrounding structure. We may have identified a low energy, peristalsis-complementary diffusion path for AZY through the periplasmic cleft that does not involve interactions with the hydrophobic trap of the putative distal site [30]. Our results suggest that multiple pathways, or residue contact pathways, may exist within the periplasmic cleft for substrates of MtrD. It is unclear whether MtrD requires multiple cycles to transport a ligand as large as AZY. It is also unclear if the presence of multiple transport ligands within the same periplasmic cleft, or in other monomers of the transporter, would aid the dissociation of AZY into the funnel domain.
1.6, Methods

1.6.1. Software

Missing residues in the 4MT1 structure of MtrD were repaired with Modeller v. 9.24 (residues 1, 494-507, 671-672, 1041-1056) [20, 31]. The complete MtrD homotrimer was built in VMD (Visual Molecular Dynamics, v 1.9.4) using the crystallographic coordinates in the 4MT1 PDB file [56]. A heterogeneous bilayer consisting of 70% POPE, 20% POPG, and 10% cardiolipin was created using the CHARMMGUI Membrane Builder with CHARMM36 topology [27, 32, 57]. The structures of the lipids used, along with their initial and final percentages, are shown in Table 1.2.1. The homotrimer was minimized to relax the modeled loops, and then embedded into the membrane using coordinates from the Orientation of Proteins in Membranes (OPM) Michigan database [36]. In simulations with NAMD, lipids were retained in the central pore of the protein.

The protein-membrane system was solvated with TIP3 water and ionized to a concentration of 0.15M NaCl using the Solvate and Autoionize plugins of VMD. All Molecular Dynamics (MD) simulations were performed with NAMD 2.12 using CHARMM36 force fields and topology unless otherwise stated. The 1.5 µs simulation of MtrD and AZY was performed with AMBER18 using the ff14SB, Lipid17, GAFF2 and TIP3p force fields employing the pmemd.cuda-DPFP molecular dynamics engine [28, 29, 58]. The CHARMM-GUI Ligand Reader and Modeller was used to format and parameterize ligands for simulations [27, 32]. PROPKA3.1 and the Henderson-Hasselbalch Equation was used to estimate ligand protonation states at a pH of 7.4. Autodock Vina 1.1.2 was used to dock AZY or SRY ligands to the drug binding domain of MtrD. Autodock Tools was used to define dock boxes [39, 59]. Bio3D was used for analysis and generation of target structures for Targeted MD simulations [60]. Protein
images were generated using UCSF ChimeraX [49]. Data analysis was performed using in-house scripts written in Tcl, R, and python. Figures were prepared with Adobe Illustrator.

1.6.2. **Ligand Docking to the Periplasmic Cleft of MtrD**

To generate a starting position for our ligands of interest, we docked azithromycin and streptomycin with the periplasmic cleft of wild-type MtrD. The structures of the MtrD substrate azithromycin (AZY) and the non-substrate streptomycin (SRY) were downloaded from PubChem and converted to 3D structures using OpenBabel v. 2.3.2 [61]. The full-length (repaired) MtrD monomer and ligands were converted to PDBQT files using AutoDock Tools v. 1.5.6 [59]. AutoDock Vina 1.1.2 was then used to dock each ligand with four overlapping boxes that encompassed the entire periplasmic cleft of MtrD (Figure 1.3.3) [39]. The docking exhaustiveness parameter was set to 128 replicates to ensure reasonable coverage of the docking regions; default exhaustiveness for Autodock Vina is 8. Docking results were processed using in-house bash scripts, producing the top 9 poses of each ligand per dock box, ranked by binding affinity.

To select ligand start sites for MD simulations, the resultant docking poses were then processed using AutoDock Tools. *The purpose of the docking experiments was to generate a plausible starting position for the ligand within the periplasmic cleft, not to evaluate individual estimated binding affinities.* Vina has an estimated standard error in calculating binding energies for small molecule redocking experiments of 2.85 kcal/mol [39]. Similar experiments for calculating the standard errors of affinity estimates for peptide- or protein-ligand complexes is expected to be much higher, due in large part to the significantly greater conformational degrees of freedom allowed for the ligand of interest. Consequently, the top 9 docking poses were evaluated according to position within the periplasmic cleft, and not to individual estimated
affinities. We found that the overlapping dock boxes produced some identical docking poses (Figure 1.3.3); from each cluster of identical poses, one representative pose was selected randomly. After the elimination of identical poses, three poses were selected for both AZY and SRY. For MD simulations with each ligand, we selected one docking pose at the Mid-Point of the cleft but within the proximal binding site – this became Start Site 1. We chose a second pose at the entrance of the periplasmic cleft – this became Start Site 2. The PDBQT files of ligands in each selected pose were converted to PDB format and “all atom” representations using Open Babel 2.3.2, since the PDB to PDBQT processing (for docking) removes all non-polar hydrogens [61].

The CHARMMGUI Ligand reader and Modeler were then used format the ligands as PDBs, to create parameter files for simulations with NAMD or AMBER, to create various protonation states if applicable (Figure 1.3.2), and to check for stereochemistry issues [27, 32]. Using available data (if possible) and analyses with Propka3.1 and OpenBabel, ligand protonation states were assessed at a physiologically relevant pH of 7.4 [61, 62]. At this pH, according to our calculations, SRY is a triple cation with only ~0.02% of molecules being double cations at pH 7.4. Therefore, we simulated the completely protonated form of streptomycin (SRY). For azithromycin (AZY), there are two ionizable sites (Figure 1.3.2). For the first ionizable site, the pKa is ~8.5-8.74, meaning that ~4.6-7.9% of AZY are unprotonated at a pH of 7.4 [37, 63]. For the second site, the pKa is 9.45, meaning that ~1% of AZY are unprotonated at this site [37]. Since it is thought that the neutral, unprotonated form of AZY may more readily diffuse through cell membranes, we included all three protonation states of AZY in our simulations.
We note also that pKa values may change depending upon the surrounding environment, and that the pKa of an amine group – there are two in AZY – is expected to decrease when moving from an aqueous to a more hydrophobic environment. This may result in higher percentages of the unprotonated form of these AZY in more hydrophobic environments. While these environments may increase the proportion of the unprotonated species of AZY, they are not expected to significantly change the protonation state of SRY.

1.6.3. **TMD Simulations of the Ligand-Free System with NAMD**

TMD simulations, and the unbiased relaxation MD simulations that preceded TMD, were performed using NAMD 2.12 with the CHARMM36 forcefield, a timestep of 2 fs, and a non-bonding cutoff of 12 Å [26, 57]. Simulations were performed with constant temperature and pressure (NPT) in a periodic cell using Langevin temperature and pressure control, and Particle Mesh Ewald electrostatics. The temperature was maintained at 310 K. As preparation for subsequent docking experiments and TMD simulations, the ligand-free system was minimized, heated to 310 K, and equilibrated for 100 ns. An equilibrated MtrD monomer was extracted from the end of the final simulation and used for docking ligands, and for aligning target structures for TMD simulations.

1.6.4. **Preparing the Protein-Ligand System**

After docking, each ligand was converted to a PDB and then uploaded, checked for structural or conformational issues, and parameterized using the CHARMM-GUI Ligand Reader and Modeller. After adding the ligand into the system and removing any overlapping water molecules using VMD, the new system was minimized, heated to 310 K, and equilibrated in unbiased MD simulations for 50 ns. Using a short TMD simulation, the $\alpha$-carbons of the equilibrated system were then guided to the starting coordinates of the 4MT1 crystal trimer.
1.6.5. **Targeted Molecular Dynamics Procedures**

To mimic a putative drug transport cycle of MtrD, TMD simulations were performed as previously described [25, 64] using target coordinates derived from structures of MtrD homologue(s) (Figure 1.1.2). In our TMD simulations, alpha carbons of the protein backbone are guided to ≤ 0.7 Å of the target coordinates. Forces are not applied to the ligand of interest, and protein sidechains move freely. When simulations were performed, no published structures of MtrD in various conformations were available, so we used structures derived from AcrB from *E. coli*, which shares a sequence identity of 48.6%, and CmeB from *C. jejuni*, which shares a sequence identity of 38.1%, with MtrD. Structures used for TMD simulations were the 4DX5 (1.9 Å) and 5NC5 (3.2 Å) structures of AcrB, and the 5LQ3 (3.5 Å) structure of CmeB (Figure 1.1.2). A comparison of e normal (“wildtype”) MtrD, MtrD<sub>CR103</sub>, and the structures used in our TMD simulations, is provided in S9 Fig. Using the structurally homologous Cα atoms of MtrD for each structure, target structures were superposed with the equilibrated MtrD-ligand system using the Bio3D module of R.

In subsequent TMD simulations on equilibrated protein-ligand systems, forces were applied using in-house *tcl* scripts in NAMD [64]. These forces were applied to selected Cα atoms of the ligand-bound protomer to gently guide the Cα atoms toward the respective target coordinates. The magnitudes of these forces were inversely proportional to the RMSD (root mean squared deviation) of the distances separating the selected Cα atoms from their target coordinates. Cα atoms were pushed to ≤ 0.7 Å of their target coordinates. Significant steric clashes were not observed between protomers, even though only one protomer was guided through conformational changes. Analysis of the protein-ligand interactions for each system were performed using *tcl* scripts in VMD and the Bio3D R module. At equal intervals throughout
each simulation, the number and characteristics of ligand-protein contacts were determined. Results are reported either for individual simulations or the averages for all twenty simulations.

We simulated three protonation states of the MtrD substrate azithromycin (AZY): AZY$_{\text{neu}}$, a neutral, unprotonated form of azithromycin; AZY$_{h1}$, a singly protonated, positively charged form of azithromycin; and AZY$_{h2}$, doubly protonated, positively charged form of azithromycin (Figure 1.3.2). As a negative control, we tested streptomycin (SRY), a known non-substrate of MtrD, and a triple cation at physiological pH. Two start sites were tested for each ligand – Site 1, in which the ligand was located in between the AP and the DP near the G-Loop, and was associating with the proximal binding site, and Site 2, in which the ligand was at the entrance of the periplasmic cleft and was associating with the foremost residues of the proximal binding site (Figure 1.3.1).

The center of mass coordinates, and ligand RMSDs from starting positions, were calculated using Tcl scripts in VMD. Protein-ligand systems were oriented such that the membrane is in the X – Y coordinate plane; therefore, positive vectorial movement along the X axis indicates movement through the periplasmic cleft towards the central region of MtrD, as would be expected during ligand transport. In contrast, negative vectorial movement along the X axis indicates movement away from the central region and towards the periplasm. Since external forces were not applied to the ligand, any movement of the ligand through MtrD should be dependent upon 1) the conformational changes of the MtrD protein, or 2) the diffusional possibilities of the transport substrate, which depend upon the protein’s conformation.

1.6.6. A Long Timescale MD Simulation of AZY-bound MtrD

Using the AMBER pmemd-cuda MD engine, we performed a GPU-accelerated, 1.5 µs simulation of the MtrD homotrimer with AZY$_{\text{neu}}$ bound at Site 1 in the periplasmic cleft [28].
The ligand was parameterized with AMBER antechamber using the GAFF2 force field, and the protein-ligand system was built in AMBER tLEAP [28, 65, 66]. Except for parameterization files, the system was identical in composition and size to the system run with NAMD, except that the membrane did not contain cardiolipin; the heterogeneous membrane (POPE/POPG only), water, and ions were maintained. Lipids were not retained within the central pore of the protein. The system was first relaxed in unbiased equilibration MD simulations for 200ns, and then allowed to run freely for a total of 1.5 µs. Analysis was performed using AMBER cpptraj, UCSF ChimeraX, the PDB2PQR server, in-house Tcl scripts with VMD or with python, and with Bio3D in R [28, 49, 56, 60, 67, 68].

1.6.7. Molecular Lipophilicity Potential Calculations

The molecular lipophilicity potential (MLP) describes the three-dimensional distribution of lipophilicity across the molecular surface of a molecule or protein. The MLP at a point in space (k) can be calculated using the following equation [48], where N is the number of molecular fragments, $F_i$ is the lipophilic contribution of each molecular fragment (i), and the distance function $f(d_{ik})$ describing the distance between the point (k) to the molecular fragment $i$:

$$MLP_k = \sum_{i=1}^{N} F_i \cdot f(d_{ik})$$

The sum of all MLP values for the molecular surfaces of the periplasmic cleft yields the Lipophilic Index (LI) of that region, defined as:

$$LI = \frac{\sum MLP^+}{\sum MLP^+ + |\sum MLP^-|} \times 100$$
MLP+ denotes regions with a positive, or lipophilic, MLP value; MLP− denotes regions with a negative, or hydrophilic, MLP value. If the fragmental contributions of the MLP+ and the MLP− of a region sum to roughly zero, then the region is classified as MLP “neutral”.

1.6.8. **Electrostatic Potential Calculations**

The electrostatic potential (EP) surfaces of the periplasmic cleft were calculated using the APBS/PDB2PQR server and visualized using ChimeraX [68-70]. All EP calculations were performed at 310 K with all other Poisson-Boltzmann parameters at default. EP calculations were performed on MtrD both in presence and absence of AZY by isolating PDB “snapshots” of the AZY-bound or ligand-free MtrD monomer from specific timepoints in the simulation; subsequent EP calculations were performed on these PDB snapshots using the APBS/PDB2PQR server.

1.6.9. **Hydration of the Solvent Accessible Surface Area (SASA)**

The percent (%) hydration of the available SASA of each ligand was calculated as follows:

\[
(1.3) \quad \% \text{Hydration of } \text{SASA} = \frac{\# H_2O \text{ at frame } N}{\# H_2O \text{ of Complete Hydration Layer}}
\]

With N being the individual frame, or timepoint, analyzed. The complete potential hydration layer describes the number of water molecules that would surround the equilibrated ligand if it was immersed freely in solvent. The complete potential hydration layer was calculated by 1) immersing each ligand into a water box, 2) neutralizing the system with 0.15 mol/L NaCl, 3) minimizing the solvated system and heating it to 310 K, and 4) simulating the ligand with free MD for 20 ns. The position of water molecules within three different radii of
AZY or SRY (radii of 3 Å, 4 Å, 5 Å, and 6 Å) were assessed to determine which radius describes a complete hydration layer around the ligand of interest. For both AZY and SRY, the optimal radius was water within 4 Å of any atom of the ligand. The number of water molecules to completely hydrate each ligand in its free-MD relaxed state was determined to be as follows: AZY_{neu}, 107 water molecules; AZY_{h1}, 103 water molecules; AZY_{h2}, 104 water molecules; SRY, 83 water molecules. The SASA was calculated using the “measure” function of VMD.

1.6.10. Modeling the MtrCDE Complex

Since the structure of MtrC remains unsolved, we used the structure of the homologous membrane fusion protein AcrA from the AcrAB-TolC RND Efflux system; AcrA shares a 44.6% sequence identity with MtrC (PDB ID 5NG5) [47]. Like AcrAB-TolC, the MtrCDE RND efflux system also assembles with a 3:6:3 stoichiometry, indicating that MtrC assembles as a hexamer [19]. A full model of MtrCDE was subsequently built using the structures of apo MtrD (4MT1), open MtrE (4MTO), and the model of MtrC [20, 50]. To assemble MtrCDE with the appropriate stoichiometry and positioning of subunits, we used the structure of fully-assembled AcrAB-TolC (5NG5) was used as a template [47]. We note that a significant flexible linker, residues 378 - 397, is missing from the 5NG5 structure of AcrA, and therefore the homology model of MtrC.
CHAPTER 2.

2.1. Introduction

Multidrug resistance (MDR) is one of most significant barriers to the successful treatment of human cancers [1]. The MDR phenomenon describes the intrinsic or acquired resistance of cancers to chemically and structurally diverse chemotherapeutics. As a mechanism of MDR, human cancers commonly overexpress membrane efflux transporters of the ATP-binding cassette (ABC) superfamily [8]. The overexpression and efflux activity of ABC transporters confer MDR to a variety of human cancers [8].

P-glycoprotein (P-gp, ABCB1) is a remarkably polyspecific ABC transporter that effluxes a multitude of structurally and chemically diverse compounds – from small, druglike molecules to large, bulky Aβ peptides [71, 72]. In humans, P-gp is primarily expressed in cells of the colon, small intestine, pancreas, kidney, and the Blood Brain Barrier – all tissues that protect sensitive areas of the body from exposure to toxic compounds and other xenobiotics [73]. P-gp harnesses the energy of ATP binding and hydrolysis to capture ‘undesirable’ molecules and extrude them into the extracellular space [25] (Figure 2.1.1). While this efflux activity is normally quite beneficial, MDR cancers commonly upregulate P-gp as an adaptation to overcome chemotherapy treatment [8]. The efflux activity of upregulated P-gp lowers the intracellular concentration of chemotherapies to sub-therapeutic levels, thereby rendering the chemotherapy ineffective, and conferring MDR on cells with upregulated P-gp expression. Inhibition of P-gp can re-sensitize MDR cancers to chemotherapeutics [3-6]; however, the search for clinically relevant P-gp inhibitors remains difficult and complex [74].
The Catalytic Efflux Cycle of P-glycoprotein (P-gp)

P-gp adopts several dynamic conformations during the catalytic efflux cycle [75, 76]. ATP binds at the cytoplasmic Nucleotide Binding Domains (NBDs, gashed grey lines), and transport substrates bind at the transmembrane Drug Binding Domains (DBDs, dashed black line). Once ATP and a transport substrate are bound, P-gp switches from an ‘Open-to-inside/cytoplasm’ conformation, to an ‘open-to-outside/extracellular space’ conformation, thereby alternating, or switching, transport substrate access from the cytoplasm to the extracellular space. It is thought that P-gp then harnesses the power of ATP hydrolysis and product dissociation to ‘re-set’ the transporter back to its starting conformation [77]. Dock boxes were designed to sample the NBDs or DBDs of human P-gp in each of these conformations. The source PDB for each structure is in parentheses.

2.2. The Search for P-gp Inhibitors

Three putative strategies to target P-gp with a small molecule inhibitor are:

1. Inhibit the transmembrane Drug Binding Domains (DBDs),
2. Inhibit the ATP binding site in the Nucleotide Binding Domains (NBDs), or
3. Allosterically inhibit P-gp at an alternative location on the protein’s solvent-accessible surface area.
As shown in Table 2.2.1, many inhibitors of P-gp have been identified, and their respective chemical and structural properties are diverse. However, many of these P-gp inhibitors are transport substrates of the pump itself, and those that were tested in clinical trials have failed [74, 78].
<table>
<thead>
<tr>
<th>Generation</th>
<th>Compound</th>
<th>MW g/mol</th>
<th>Clinical Trial Outcome</th>
<th># RO5 Violations</th>
<th>TPSA (Å²)</th>
<th>Consensus Log P₂oct/wat</th>
<th>Consensus LogS</th>
<th>Predicted P-gp Substrate</th>
<th>Also a P-gp Substrate</th>
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<tr>
<td>1st Gen</td>
<td>Verapamil</td>
<td>455</td>
<td>Fail</td>
<td>0</td>
<td>64</td>
<td>4.45</td>
<td>Moderate</td>
<td>Yes</td>
<td>✓</td>
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<tr>
<td></td>
<td>Reserpine</td>
<td>609</td>
<td>Fail</td>
<td>2</td>
<td>118</td>
<td>3.52</td>
<td>Poor</td>
<td>Yes</td>
<td>✗</td>
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<tr>
<td></td>
<td>Cyclosporin A</td>
<td>1203</td>
<td>Fail</td>
<td>2</td>
<td>279</td>
<td>2.38</td>
<td>Poor</td>
<td>Yes</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
<td>324</td>
<td>Fail</td>
<td>0</td>
<td>46</td>
<td>2.81</td>
<td>Soluble</td>
<td>No</td>
<td>✓</td>
</tr>
<tr>
<td>2nd Gen</td>
<td>Valspodaar</td>
<td>1215</td>
<td>Fail</td>
<td>2</td>
<td>276</td>
<td>2.26</td>
<td>Poor</td>
<td>Yes</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td>Elacridar</td>
<td>564</td>
<td>Fail</td>
<td>1</td>
<td>93</td>
<td>4.93</td>
<td>Poor</td>
<td>No</td>
<td>✓</td>
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<tr>
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<td>Dexverapamil</td>
<td>455</td>
<td>Fail</td>
<td>0</td>
<td>64</td>
<td>4.45</td>
<td>Moderate</td>
<td>Yes</td>
<td>✗</td>
</tr>
<tr>
<td>3rd Gen</td>
<td>Tariquidar</td>
<td>647</td>
<td>Fail</td>
<td>1</td>
<td>111</td>
<td>5.2</td>
<td>Poor</td>
<td>No</td>
<td>✗</td>
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<tr>
<td></td>
<td>Zosuquidar</td>
<td>528</td>
<td>Fail</td>
<td>1</td>
<td>49</td>
<td>4.64</td>
<td>Poor</td>
<td>No</td>
<td>✓</td>
</tr>
<tr>
<td>Undesignated Generation</td>
<td>Clarithromycin</td>
<td>748</td>
<td>Fail</td>
<td>2</td>
<td>183</td>
<td>2.37</td>
<td>Moderate</td>
<td>Yes</td>
<td>✓</td>
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<td>Ritonavir</td>
<td>721</td>
<td>Fail</td>
<td>2</td>
<td>202</td>
<td>5.04</td>
<td>Insoluble</td>
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<td>Fail</td>
<td>3</td>
<td>105</td>
<td>4.74</td>
<td>Poor</td>
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<td>Dronedarone</td>
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<td>Fail</td>
<td>1</td>
<td>97</td>
<td>6.05</td>
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<td>Yes</td>
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<td>0</td>
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<td>4.47</td>
<td>Poor</td>
<td>No</td>
<td>✓</td>
</tr>
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<td>Ranolazine</td>
<td>428</td>
<td>Fail</td>
<td>0</td>
<td>74</td>
<td>2.41</td>
<td>Soluble</td>
<td>Yes</td>
<td>✗</td>
</tr>
<tr>
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<td>Ticagrelor</td>
<td>523</td>
<td>Fail</td>
<td>1</td>
<td>164</td>
<td>2.43</td>
<td>Moderate</td>
<td>Yes</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>531</td>
<td>Fail</td>
<td>1</td>
<td>69</td>
<td>3.55</td>
<td>Moderate</td>
<td>No</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Tacrolimus</td>
<td>804</td>
<td>Fail</td>
<td>2</td>
<td>178</td>
<td>3.71</td>
<td>Poor</td>
<td>Yes</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 2.2.1. Known P-gp Inhibitors and Predicted Chemical Properties.  
Here we present a non-exhaustive list of known P-gp inhibitors and their selected chemical properties [71, 79]. Clinical trial outcomes were adapted from [74, 78]. Compounds were evaluated using the SWISS-ADME server [53]. RO5: Rule of 5, MW: Molecular Weight, TPSA: Topological Polar Surface area, Consensus LogP: Average of 5 calculations of LogP₂oct/wat, Consensus LogS: Average of 3 calculations to determine aqueous solubility – Insoluble, Poorly soluble (Poor), Moderately Soluble (Moderate), Soluble. “Predicted P-gp Substrate” - predicted by SWISS-ADME to be a transport substrate of P-gp. “Known P-gp Substrate” – In addition to being P-gp inhibitors, these molecules are: (√) known transport substrates of P-gp, (✗) known non-transport substrates of P-gp, (?) unknown whether it is a transport substrate of P-gp [73, 80, 81]. If the molecule is designated in a specific generation of P-gp inhibitors - First Generation P-gp inhibitors¹, Second generation P-gp inhibitors², Third generation P-gp inhibitors³.
P-gp inhibitors have been divided into three groups: First Generation, Second Generation, and Third Generation. First Generation (1\textsuperscript{st} gen) inhibitors (Table 1.2.1, \textsuperscript{1}) tend to be substrates of other ABC transporters or enzymes, and bind P-gp with relatively low affinities [74]. The best-characterized 1\textsuperscript{st} gen inhibitor is likely Verapamil – an inhibitor and transport substrate of P-gp that failed in clinical trials due to cardiac toxicity [78]. Second Generation (2\textsuperscript{nd} gen) P-gp inhibitors (Table 1.2.1, \textsuperscript{2}) typically exhibit greater specificity to P-gp than the 1\textsuperscript{st} gen molecules, but still interact with, and are substrates of, other pharmacologically important enzymes like CYP 3A4 or other ABC transporters [74]. As shown with Dexverapamil, a variant of the 1\textsuperscript{st} gen Verapamil, these 2\textsuperscript{nd} gen inhibitors also tended to fail in clinical trials. It is thought that both 1\textsuperscript{st} and 2\textsuperscript{nd} gen P-gp inhibitors fail because they are A) non-specific to P-gp, and thus cause significant off-target interactions, and/or B) are transport substrates of P-gp itself, and therefore must be administered at a relatively high dosage to reach a therapeutic concentration intracellularly [74]. Thus, the Third Generation (3\textsuperscript{rd} gen) of P-gp inhibitors were expected to address these issues. 3\textsuperscript{rd} gen inhibitors like Tariquidar and Zosuquidar are not transport substrates of P-gp [82] and bind with improved specificities [74]. However, despite these improvements, these 3\textsuperscript{rd} gen inhibitors failed in clinical trials (Table 2.2.1) [83, 84].

Why did these 3\textsuperscript{rd} gen P-gp inhibitors fail in clinical trials, despite promising outcomes in \textit{in vitro} and biophysical studies? A 2021 study of BCRP by Antoni et al. provides a clue. BCRP (Breast Cancer Resistance Protein, \textit{ABCG2}) is an ABC transporter that also confers MDR to human cancers, and its inhibitors and substrates share some overlap with those of P-gp [8]. Antoni et al. note that the Tariquidar-related BCRP inhibitor UR-MB108 exhibits poor aqueous solubility, and that this characteristic is correlated with poor outcomes in drug development [85]. For example, an orally-administered medication must dissolve in aqueous gastrointestinal fluids
in order to be efficiently absorbed through the small intestine [86]. Even if the drug is administered intravenously, aqueous solutions are the solvent of choice [87]. Furthermore, poorly soluble compounds have been shown to precipitate \textit{in vivo} and cause significant toxic health effects [88].

Our analysis with SWISS-ADME reveals that many known P-gp inhibitors are poorly soluble in aqueous solution (Table 2.2.1, “Poor”), with the next most populated category being moderately soluble (Table 2.2.1, “Moderate”). We have found that the aqueous solubility predictors are generally correct in practice. Therefore, we hypothesize that 3\textsuperscript{rd} gen inhibitors may have failed in clinical trials due, in part, to poor aqueous solubility along with off-target effects and associated toxicities [74, 78]. UR-MB108 is a BCRP inhibitor and a derivative of the P-gp inhibitor Tariquidar, and UR-MB108 exhibits poor solubility in aqueous solution [85]. Notably, when Antoni et al. designed UR-MB108 variants with a focus on increased hydrophilicity, these variants were more soluble and stable than the original molecule, and still inhibited BCRP in the low nano-molar range [85]. It should be acknowledged that this approach may not work for all P-gp or BCRP inhibitors. However, the aqueous solubility of the compounds, specifically the underlying molecular characteristics that contribute to hydrophilicity, are becoming increasingly important.

Many 1\textsuperscript{st} and 2\textsuperscript{nd} gen P-gp inhibitors are transport substrates of the pump itself, and these molecules must, by definition, interact with the DBDs of the protein (Table 2.2.1). Like many 1\textsuperscript{st} and 2\textsuperscript{nd} gen P-gp inhibitors before them, 3\textsuperscript{rd} gen inhibitors have also been shown to bind at the DBDs of P-gp [89, 90]. Since these P-gp inhibitors are preferentially interacting with the large, hydrophobic DBDs of these ABC transporters, Antoni et al. suggest that these molecules are ‘predisposed’ to be poorly soluble in aqueous solutions, and thereby perform poorly \textit{in vivo} [89,
Based upon the best available evidence and the outcomes of clinical trials, we suggested in 2014 that the DBDs of P-gp are no longer a promising target for small molecule inhibitors and that the cytoplasmic NBDs of P-gp are a more promising target [3].

We have shown that small molecule inhibitors targeting the NBDs of P-gp can perturb ATP binding and reverse MDR in P-gp overexpressing human cancers [3, 4]. These initial inhibitors were identified in screens against one structure of human P-gp [64], and as shown in Figure 2.1.1, P-gp samples a variety of dynamic conformations during the substrate efflux process [25]. In this work, we sought to identify novel P-gp inhibitors with an enhanced structure-guided approach that incorporates the transporter’s dynamic nature into the virtual screening process. Using subtractive, massively parallel docking screens and a combination of cell-based assays, we have identified 6 novel P-gp inhibitors that reverse MDR in human cancers and are not transport substrates of the pump itself. We report a hit rate of 13% for P-gp inhibitors overall, and a hit rate of 9% for P-gp inhibitors that are not transport substrates of the pump itself.

2.3. Results

First, a diverse library of compounds was screened virtually against dynamic P-gp targets in an iterative process, with each P-gp target modeling a putative conformation of the catalytic efflux cycle [25, 64]. Hits from computational screens were then assessed for their ability to reverse MDR using two sets of paired human carcinoma lines – two chemotherapy resistant, P-gp overexpressing lines, and two chemotherapy-sensitive, non-P-gp overexpressing lines. Top hits were tested for inherent toxicity using a non-cancerous human cell line. Compounds were then assessed as transport substrates of P-gp using LC-MS/MS accumulation assays. Lastly, we
analyzed and discussed the common characteristics of our P-gp inhibitors and identified 6 novel drug candidates for optimization. An overall workflow is shown in Figure 2.3.1.
Figure 2.3.1. **Protocol to Identify and Test Putative Inhibitors of P-gp.**

Here we present an overall workflow of the combined protocols for *in silico* and *in vitro* screening. In the first phase of the project, we use *in silico* techniques to iteratively screen compounds in millions of virtual docking experiments. Our docking studies target the NBDs of P-gp, not the DBDs. In phase two, we use *in vitro* techniques to test our compounds for 1) the ability to reverse MDR in P-gp overexpressing cancer, 2) off-target toxicity, 3) potential as transport substrates of P-gp, and 4) to potentiate toxicity of chemotherapy in non-P-gp overexpressing cancer. Figure created with the Biorender program (www.biorender.com).
2.3.1. Screening of Putative P-gp Inhibitors via Molecular Docking

Over 100,000 compounds from the ZINC clean, druglike library were iteratively screened by James McCormick against dynamic P-gp targets using Autodock Vina (McCormick 2017, unpublished) [25, 39, 64]. This subset of the ZINC library contains 13.2 million molecules that meet the following criteria:

1. Lipinski’s Rule of Five (RO5) [91], i.e. molecules with the following characteristics -
   a. A molecular weight between 150 and 500 Daltons.
   b. $\text{LogP}_{\text{oct/wat}} \leq 5$.
   c. $\leq 5$ H-bond donors.
   d. $\leq 10$ H-bond acceptors.
2. Topological Polar Surface Area is $\leq 150 \text{ Å}^2$.
3. Number of Rotatable Bonds is $\leq 7$.
4. “Clean” – aldehydes and thiols are filtered out.

As targets for molecular docking screens, we used a homology model of human P-gp and known conformations of other homologous ABC-transporters, with each structure representing a putative stage in the catalytic efflux cycle [25, 64]. These conformations were isolated from TMD experiments performed as in [25] and are shown in Figure 2.3.2 and labeled with the PDB ID of the respective source structure. The 4KSB and 3B5X structures are ‘open-to-the-inside’ conformations, in which P-gp is ready to capture substrates for efflux across the cell membrane [76, 92]. In ‘open-to-the-inside’ conformations, the NBDs are disengaged, and the DBDs are open to the cytoplasm. The ‘Transition’ structure is a conformational intermediate between 4KSB and 3B5X and was extracted from TMD simulations; the position of the NBDs is shown in Figure 2.3.2 by a dashed grey line. The 2HYD and 3B5Z structures are ‘open-to-the-outside’
conformations that should occur near the end of the catalytic efflux cycle [76, 93]. In ‘open-to-the-outside’ conformations, the NBDs of P-gp are engaged, and the DBDs are open to the extracellular space.

As shown in Figure 2.3.2, dock boxes were designed to sample the NBDs and/or DBDs of P-gp in a variety of conformations, the goal being to identify molecules with high estimated affinities to the NBDs, and low estimated affinities to the DBDs.

Figure 2.3.2 Docking Experimental Compounds to the NBDs and DBDs of P-gp. Docking and TMD experiments were performed by James McCormick. Here we show the structures of P-gp used for the virtual screening of putative P-gp inhibitors. 4KSB, 3B5X, 2HYD and 3B5Z are all conformations of P-gp homologs, whereas ‘Transition’ is a conformational intermediate isolated from TMD simulations [25]. The approximate position of the NBDs in the transition structure is shown by a dashed grey line, indicating that P-gp is slightly more closed to the cytoplasm in Transition than it is in the 4KSB structure. Boxes denote the actual Dock Boxes used in virtual screens with Autodock Vina. Boxes A-D) sample the NBDs of 4KSB, Transition, 2HYD and 3B5Z, whereas boxes 1-3) sample the DBDs of 4KSB, Transition, and 3B5X.
After each molecule was screened against the entire array of P-gp targets (Figure 2.3.2), we calculated the ratio between the estimated binding affinity to the NBDs (target) and the DBDs (off target). An NBD/DBD ratio less than 1 indicates a greater estimated affinity to the DBDs of P-gp, whereas an NBD/DBD ratio greater than 1 indicates a greater estimated affinity to the NBDs relative to the DBDs. We hypothesized that compounds with a low NBD/DBD ratio, and thus a greater predicted preference for the DBDs over the NBDs, would be more likely to be transport substrates of P-gp, a characteristic we desired to minimize. Molecules with a low NBD/DBD ratio were eliminated during each iterative round of screening. After the final docking screen, the top 100 molecules against each P-gp target structure were subjected to quantitative structure-activity relationship (QSAR) predictions using the Online chemical database (OCHEM). These OCHEM analyses also provided predictions of inherent toxicity. The QSAR data, selected chemical properties, and predicted docking locations were used to select a subset of 67 compounds for purchase and testing. Since this work is an extension of previous work by us, compound numbering begins with #58. Experimental compounds are numbered 58 to 124 in order of arrival to the laboratory [56].

2.3.2. Putative P-gp Inhibitors Overcome MDR in P-gp Overexpressing DU145-TXR Cells

Compounds 58 to 124 were tested for their ability to re-sensitize the multidrug-resistant, P-gp overexpressing DU145-TXR prostate carcinoma line to the chemotherapeutic, Paclitaxel (PTX) [94]. DU145-TXR cells greatly overexpress P-gp and exhibit a 34-fold increase in the IC$_{50}$ of PTX relative to the non-P-gp overexpressing, chemo-sensitive DU145 parental cells [94]. In assays with P-gp overexpressing, MDR human cancer lines, compounds were screened for the ability to re-sensitize MDR cancer cells to PTX. Since PTX is a transport substrate of P-gp, “re-sensitization” of MDR cancer cells to PTX suggests that the compound may be interfering with
P-gp-mediated transport of PTX. If a compound is intrinsically toxic to MDR cancer cells, then it is more likely that the compound is acting through an alternate – in this case, undesirable – mechanism. If compounds are toxic to MDR cancer cells, the compound’s intrinsic toxicity should be observable in both the parental and MDR lines as decreased cell viability when treated with the compound alone.

DU145-TXR cells were exposed to the experimental compounds at a concentration of 15 µM in the presence or absence of 500 nM PTX for 48 hours, after which cell viability was assessed. Compounds 58 to 98 were initially assessed using the Resazurin cell viability assay by Courtney Follit [5] (Follit 2017, unpublished). From this first test, compounds 60, 61, 66, 68, 70, 71, 78, 96, and 97 were shown to reverse MDR, resulting in a ≥ 30% re-sensitization of DU145-TXR cells to PTX without exhibiting intrinsic toxicity.

For the testing of compounds 99 to 124, cell viability was assessed after 48 hours using the MTT cell viability assay [95]. As positive controls for P-gp inhibition, we included the P-gp inhibitors Tariquidar (TQR) and Verapamil (VPL); as negative controls for P-gp inhibition, we included the BCRP inhibitor Ko143 and the vehicle (DMSO) control. To re-evaluate the testing of compounds 58-98 with MTT assays, the top 9 compounds from the initial screen (compounds 60, 61, 66, 68, 70, 71, 78, 96, and 97) were re-tested. Compounds 59 and 89 were also included as internal negative controls for the re-assessment of top hits from the Resazurin assays since neither compound reversed MDR in the initial screen against DU145-TXR. Results of the entire screen are shown in Figure 2.3.3.

Interestingly, when re-tested with MTT assays, compounds 60, 66, 68, and 71 did not perform as expected. These compounds did not reverse MDR, and in the case of compounds 68 and 71, exhibited unexpected levels of intrinsic toxicity. Notably, the DU145-TXR cells
responded as expected to the included control compounds - TQR, VPL, and Ko143. Furthermore, compounds 59 and 89 did not reverse MDR when re-tested, confirming that these compounds did not re-sensitize DU145-TXR cells to chemotherapy. Considering these data, it is possible that the stocks of compounds 60, 66, 68, and 71 had decayed in storage over time (these compounds were purchased in 2016). However, to account for the possibility that the disparity in re-sensitization could be due to methodological differences between the two viability assays, these compounds were included in subsequent testing, as they were originally identified as agents of MDR reversal (Follit 2017, unpublished). Here we also note that compounds 71, 103, 108, and 120 were difficult to dissolve properly in DMSO, and prior to usage in cell-based assays, these compounds were sonicated to enhance solvation (15 minutes at 27 °C). Despite these efforts, treatment with these compounds often resulted in significant precision problems as reflected in the standard error measurements.

Summarizing the results of these assays, compounds 60, 61, 66, 68, 70, 71, 78, 79, 96, 97, 101, 103, 111, and 122 resulted in ≥ 30% re-sensitization of DU145-TXR cells to PTX and did not exhibit significant intrinsic toxicity to these cells. Intrinsic toxicity is defined as a ≤ 30% reduction in viability of human cancer cells when treated with compound alone. Compound 124 performed close to the re-sensitization cutoff and exhibited minimal intrinsic toxicity, and thus was included in the next round of trials. Of the tested compounds, 15/67 were promising candidates, and the remaining compounds were eliminated from further consideration as targeted inhibitors of P-gp.
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Figure 2.3.3. **Testing of Experimental Compounds Against the DU145-TXR Cell Line.**
A total of 67 experimental compounds were purchased from *in silico* screens for testing *in vitro*. DU145-TXR cells were exposed to experimental compounds in the presence or absence of 500 nM of the P-gp substrate (and chemotherapeutic) Paclitaxel (PTX). Experimental compounds at 15 µM. Tariquidar (TQR) and Verapamil (VPL) were used as positive controls for inhibition of P-gp, whereas Ko143 was used as a negative control for inhibition of P-gp. TQR and VPL were used at 15 µM, and Ko143 was used at 1 µM. Cells were exposed to the experimental treatments for 48 hours, after which cell viability was assessed using the MTT viability assay. Data represent the mean ± one standard deviation from the mean, n = 8, from at least two trials.

2.3.3. **Putative P-gp Inhibitors do not Outperform Chemotherapy alone against DU145 Cells**

The 16 compounds identified from the screens against DU145-TXR cells were tested for their ability to potentiate toxicity of PTX against the chemotherapy sensitive, non-P-gp-overexpressing DU145 cell line [94]. Quantitative One-Step qPCR assays reveal that the MDR DU145-TXR cancer cells overexpress P-gp by 14,000 fold compared to the PTX-sensitive DU145 parental line [96]. DU145 cells express very low – but detectable – amounts of P-gp [96], and are highly sensitive to PTX [94].
Consequently, in assays against DU145 and other non-P-gp overexpressing cancer cells, we screen for compounds that do not greatly enhance the toxicity of PTX. If a compound significantly enhances the toxicity of PTX against these non-P-gp overexpressing, PTX-sensitive cancer cells, then the compound is less likely to exert its effects through inhibition of P-gp and is more likely to act through an alternate (undesired, in this case) mechanism. We note that these criteria do not apply to the P-gp overexpressing DU145-TXR cell line.

DU145 cells were exposed to the experimental compounds at a concentration of 15 µM in the presence or absence of 500 nM PTX, after which cell viability was assessed using MTT assays [95]. Including the vehicle (DMSO) treated control, we also included Tariquidar (TQR), Verapamil (VPL) and Ko143 control treatments. Results are shown in Figure 2.3.4. In Figure 2.3.4 a dashed line marks the viability of DU145 cells treated with PTX alone – this is the point of reference, ideal P-gp inhibitors should not greatly enhance the toxicity of PTX below this point. Compounds 60, 70, 78, 96, 97, 101, 103, 111, 122, and 124 (10/15) resulted in insignificant or acceptable re-sensitization of DU145 cells to PTX. Except for compound 122, these compounds did not exhibit intrinsic toxicity. Notably, treatment with compounds 66, 68, 79, and VPL alone resulted in significant intrinsic toxicity. Since compounds 66 and 68 performed poorly when re-tested against the DU145-TXR cell line, these two compounds were not included in subsequent trials with the A2780 and A2780-ADR cell lines.
2.3.4. **Putative P-gp Inhibitors are not Specific to the DU145-TXR or DU145 Cell Lines**

To test if the effects of our putative P-gp inhibitors were specific to the DU145 and DU145-TXR lines, we screened our compounds against a second pair of human carcinoma lines – the A2780-ADR P-gp overexpressing ovarian cancer line, and the parental, non-P-gp overexpressing A2780 line. A2780-ADR greatly overexpresses P-gp and is resistant to Doxorubicin, a chemotherapeutic and a P-gp substrate [5]. For assays with A2780 and A2780-ADR, we tested the top 10 compounds from our screens against the DU145-TXR and DU145 lines.
cell lines – compounds 70, 78, 96, 97, 101, 103, 111, 122 and 124. TQR, VPL and Ko143 control treatments were included as well. As internal negative controls for P-gp inhibition, we included two compounds that did not reverse MDR in DU145-TXR cells – compounds 59 and 89. The results of our screens against the A2780 and A2780-ADR carcinoma lines are shown in Figure 2.3.5. Of the 10 compounds tested, 9 (70, 78, 96, 97, 101, 103, 111, 122, and 124) reverse MDR in the A2780-ADR cells. Of these 9 compounds, none outperformed PTX alone against the A2780 cells except compound 79. Considering these data, compound 79 was excluded from further testing due to its demonstrated intrinsic toxicity.
Figure 2.3.5 **Compound Testing Against the A2780 and A2780-ADR Cell Lines.**
The top 10 compounds from screens with DU145 and DU145-TXR were tested against two additional carcinoma lines – A) the chemotherapy-resistant, P-gp overexpressing A2780-ADR line and B) the chemotherapy-sensitive, non-P-gp overexpressing parental A2780 line. Cells were exposed to compounds in the presence or absence of 500 nM Paclitaxel (PTX) for 48 hours. Cell viability was assessed using the MTT viability assay. Data represent the mean ± one standard deviation from the mean, n = 8, from at least two trials. In B) a black dashed line marks the survival of A2780 cells when exposed to PTX alone; putative P-gp inhibitors are not expected to outperform (i.e. result in a lower overall survival) PTX alone against A2780 cells.

2.3.5. **In-Silico Identified P-gp Inhibitors are not Transport Substrates of P-gp**

We sought to identify P-gp inhibitors that are not transport substrates of the pump itself, and this goal was the primary focus behind the subtractive docking methods shown in Figure 2.3.2. To determine whether our compounds are transport substrates of P-gp, we used LC-MS/MS accumulation assays as described in [5]. P-gp overexpressing DU145-TXR cells were exposed to 5 µM compound in the presence or absence of 500 nM TQR for 2.5 hours at which point the relative intracellular accumulation of each compound was assessed using LC-MS/MS (see Methods). If the intracellular accumulation of compound increased significantly in the
presence of TQR (which at the concentration used should nearly completely inhibit P-gp catalysis), then the compound is likely to be a transport substrate of P-gp. If the observed difference in intracellular accumulation between TQR treated and TQR-untreated cells was insignificant, then the compound is unlikely to be a transport substrate of P-gp.

We assayed 13 compounds identified by our initial screens against the DU145-TXR cells (Figure 2.3.3). Daunorubicin, a chemotherapeutic and transport substrate of P-gp, was included as a positive control (Daunorubicin is a known substrate of P-gp). Compound 74 was included as an example of an experimental compound that did not perform well in assays against DU145-TXR cells but was shown to modulate the ATPase activity of P-gp in ATPase assays performed by Gang Chen (personal communication). The raw quantification data from our LC-MS/MS experiments are shown in Table 2.3.1. The processed and interpreted data are shown in Table 2.3.2.
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Table 2.3.1. Normalized Ratio of Compound to Internal Standard using LC-MS/MS. Here we show the raw data from our LC-MS/MS trials. DU145-TXR cells were exposed to 5 µM compound in the presence (+TQR) or absence (-TQR) of 500 nM Tariquidar (TQR) as described in [5]. Samples were prepared in triplicate and two or three independent trials were performed; data represent the mean ± one standard deviation (Std. Dev). In contrast to the methods used in [5], we note that these LC-MS/MS trials measured the Normalized Ratio of Analyte (i.e. compound) to the Internal Standard (i.e. unique performance of Mass Spectrometer on date of analysis); thus the data represent the relative quantification of compound per sample, variability between independent trials is unsurprising (see Methods).
As shown in Table 2.3.2, 9 of the 13 compounds (60, 66, 70, 71, 78, 96, 97, 101, and 111) are unlikely to be transport substrates of P-gp. Of these 9 ‘unlikely substrates’, three (60, 66, and 71) were eliminated during testing against the DU145 parental line (Figure 2.3.4). The remaining six compounds (70, 78, 96, 97, 101, 111) performed quite well in assays against the A2780 and A2780-ADR cell lines (Figure 2.3.5). Even though compounds 122 and 124 were identified as potential substrates of P-gp, these inhibitors also performed well in assays with A2780 and A2780-ADR. Compound 103, which was also identified as a transport substrate, re-sensitized A2780-ADR cells to PTX, but was observed to be intrinsically toxic to both A2780 and A2780-ADR cells.
Table 2.3.2. Intracellular Accumulation of Compounds Measured by LC-MS/MS.
The intracellular accumulation of each experimental compound (5 µM) was assessed in P-gp overexpressing DU145-TXR cells using LC-MS/MS in the presence and absence of 500 nM Tariquidar (TQR). Samples were prepared in triplicate and in two independent trials unless otherwise indicated; n = 6 total. Compounds 61, 66, 74, and 103 were assayed in three independent trials, n = 9 total. Significance was determined using a Student’s T test of the mean; P > 0.05 = N.S., P < 0.05 = *, P < 0.01 = **, P < 0.001 = ***, P < 0.0001 = ****. In addition to the final quantified sample, DAU was included as a qualitative internal control for each assay performed. Raw data are shown in Table 2.3.1.

2.3.6. In-Silico Identified P-gp Inhibitors are not Toxic to Non-Cancerous Human Cells

Considering data from MTT assays and LC-MS/MS accumulation assays, a subset of compounds was selected for toxicity testing against the non-cancerous, human HFL1 cells.

Compounds were assessed for intrinsic toxicity against HFL1 cells at a concentration of 15 µM over 48 hours, after which cell viability was assessed using MTT cell viability assays. If
treatment with a compound resulted in a ≤ 20% reduction in cell viability, then the compound was considered within the acceptable limits of toxicity. As a positive control for inherent toxicity, we included the chemotherapeutic, Daunorubicin (also a substrate of P-gp); as negative controls for inherent toxicity, we included the P-gp inhibitors Verapamil (VPL) and Tariquidar (TQR). As shown in Figure 2.3.6, nine tested compounds (70, 78, 96, 97, 101, 103, 111, 122 and 124) were classified as either ‘non-toxic’ or ‘within acceptable limits’ of toxicity in assays with HFL1 cells. In addition to being non-toxic, compounds 103, 122 and 124 were observed to likely be transport substrates of P-gp; the remaining non-toxic compounds – 70, 78, 96, 97, 101 and 111 – are unlikely to be transport substrates of P-gp (Table 2.3.2).
2.3.6. **Evaluation of Inherent Toxicity of Potential P-gp Inhibitors.**

The top 16 compounds from screens against DU145 and DU145-TXR were tested against the non-cancerous HFL1 cell line. HFL1 cells were exposed to 15 µM of experimental compound. Tariquidar (TQR) and Verapamil (VPL) were used as negative controls for inherent toxicity, whereas Daunorubicin (DAU) – a chemotherapeutic and substrate of P-gp – was used as a positive control for inherent toxicity. Cells were exposed to the experimental treatments for 48 hours, after which cell viability was assessed using the MTT viability assay. Data represent the mean ± one standard deviation from the mean, n = 8, from at least two trials. A red dashed line marks 80% viability of DMSO-treated cells.

![Graph showing evaluation of inherent toxicity](image)

2.3.7. **Experimental P-gp Inhibitors Enhance the Intracellular Retention of a P-gp Substrate**

The chemotherapeutic Daunorubicin (DAU) is a transport substrate of P-gp and is intrinsically fluorescent (excitation/emission at 488 nm / 575 nm). We tested our experimental inhibitors for their ability to enhance the fluorescence of DAU in the DU145-TXR cell line. Assays were performed as described in [5] with minor modifications. Cells were treated with 10 µM of experimental compound in the presence or absence of 10 µM DAU. Cells were incubated with these respective treatments for 2 hours, after which cells were washed and lysed for analysis. The total fluorescence of each well was then measured using a Cytation 5 fluorescence
plate reader. Results are shown in Figure 2.3.7 and Table 2.3.3. Treatment with compounds 70, 96, 97, 101, 103, VPL, and TQR significantly enhanced the fluorescence of DAU in DU145-TXR cells. As expected, compound 59 did not enhance fluorescence of DAU in either cell line, but also as expected, treatment with TQR and VPL did significantly enhance total fluorescence of DAU.

Unexpectedly, compounds 78 and 111 did not significantly enhance DAU fluorescence in DU145-TXR cells. Several factors could have contributed to these unexpected results – first, the concentration of experimental compound used, and second, the incubation time used. These DAU accumulation experiments used a concentration of 10 µM compound and an incubation time of 2 hours. For comparison, our MTT assays use a compound concentration of 15 µM and an incubation time of 48 hours; our LC-MS/MS assays use a concentration of 5 µM and an incubation time of 2.5 hours.

Both 78 and 111 were identified as Paclitaxel (PTX) sensitizers against DU145-TXR and A2780-ADR at a concentration of 15 µM (Figure 2.3.3 and Figure 2.3.5). Thus, it is possible that compounds 78 and 111 are ineffective as P-gp inhibitors at concentrations less than 15 µM. If so, these compounds are still good candidates for lead optimization efforts with a focus on increased specificity to P-gp, particularly since both compounds were not toxic to non-cancerous HFL1 cells (Figure 2.3.6). However, specifically with compound 78, it is also possible that the shorter incubation time does not allow sufficient time for 78 to cross the cell membrane.

Neither 78 nor 111 were identified as a transport substrate of P-gp in LC-MS/MS assays (Table 2.3.2). A closer examination of the initial LC-MS/MS data used to draw these conclusions reveals, however, that compounds 78 and 111 exhibited remarkably low normalized analyte ratios in the presence and absence of TQR (Table 2.3.2). For example, the normalized analyte
ratio (hereafter referred to as “ratio”) of Daunorubicin (DAU) in the absence of TQR is 0.014 ± 0.001 or 0.017 ± 0.001, respectively. Upon the addition of TQR, the ratio, which serves as a relative quantification of intracellular DAU, increases to 0.216 ± 0.002 (15-fold increase) and 0.232 ± 0.016 (14-fold increase), respectively. DAU is a “good” substrate of P-gp. In comparison, the observed ratio of compound 78 is 0.001 ± 0.000 regardless of the presence of TQR (Table 2.3.2). These values are the lowest observed for any compound in our LC-MS/MS trials, and suggest that compound 78 was almost undetectable in cell lysates from both samples. In combination with our DAU fluorescence results, these data suggest that compound 78 is unable to cross the cell membrane – in sufficient concentrations to inhibit P-gp – in under 2.5 hours. When a longer incubation time is used, as with our MTT assays, compound 78 is quite effective in reversing MDR (Figure 2.3.3, Figure 2.3.5). Consequently, lead optimization efforts for compound 78 should also explore modifications to enhance diffusion across the cell membrane, in addition to enhancing specificity and increasing affinity towards P-gp.

Additionally, we note that compound 103 was identified as a transport substrate of P-gp in LC-MS/MS assays (Table 2.3.2) and was also found to be intrinsically toxic to non-cancerous HFL1 cells (Figure 2.3.6). Since treatment with compound 103 resulted in a 4-fold accumulation of DAU (Table 2.3.3), it is possible that compound 103 is a competitive substrate and inhibits the pump via competition for binding with DAU at the pump-substrate site of P-gp, similar to the well characterized competitive inhibition mechanism of Verapamil [74].
Figure 2.3.7. **Change in DAU Fluorescence in the Presence of Experimental Compounds.**
Here we show the fold fluorescence of the P-gp substrate Daunorubicin (DAU) in the presence or absence of experimental compounds or known P-gp modulators. Cells were treated with 10 µM compound in the presence or absence of 10 µM DAU, after which cells were washed and lysed; the resultant DAU fluorescence was measured on the Cytation 5 (excitation/emission 488 nm / 575 nm). The resultant DAU fluorescence is expressed as a fold change relative to the fluorescence of DU145-TXR cells treated with DAU alone. The P-gp inhibitors VPL and TQR were included as positive controls for P-gp inhibition, and compound 59 was included as a negative control for P-gp inhibition. N = 9, with three samples per trial, three trials. Statistical significance determined using GraphPad Prism, Student’s T test of the mean. Significance was determined using a Student’s T test of the mean; P > 0.05 = N.S., P < 0.05 = *, P < 0.01 = **, P < 0.001 = ***, P < 0.0001 = ****.
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<tr>
<td><strong>DAU + 97</strong></td>
<td>1.9 ± 0.4</td>
<td>P &lt; 0.0001</td>
<td>****</td>
</tr>
<tr>
<td><strong>DAU + 101</strong></td>
<td>2.3 ± 0.4</td>
<td>P &lt; 0.0001</td>
<td>****</td>
</tr>
<tr>
<td><strong>DAU + 103</strong></td>
<td>4.5 ± 2.6</td>
<td>P = 0.001</td>
<td>**</td>
</tr>
<tr>
<td><strong>DAU + 111</strong></td>
<td>1.0 ± 0.2</td>
<td>P = 0.75</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>DAU + VPL</strong></td>
<td>6.3 ± 1.0</td>
<td>P &lt; 0.0001</td>
<td>****</td>
</tr>
<tr>
<td><strong>DAU + TQR</strong></td>
<td>6.5 ± 1.6</td>
<td>P &lt; 0.0001</td>
<td>****</td>
</tr>
</tbody>
</table>

Table 2.3.3. **Fold Intracellular Accumulation of DAU in DU145-TXR Cells.**

Here we show the data presented in Figure 2.3.7, mean ± one standard deviation of the fold fluorescence of the P-gp substrate Daunorubicin (DAU) in the presence or absence of experimental compounds or known P-gp modulators. Cells were treated with 10 µM compound in the presence or absence of 10 µM DAU, after which cells were washed and lysed; the resultant DAU fluorescence was measured on the Cytation 5 (excitation/emission 488 nm / 575 nm). The resultant DAU fluorescence is expressed as a fold change relative to the fluorescence of DU145-TXR cells treated with DAU alone. The P-gp inhibitors VPL and TQR were included as positive controls for P-gp inhibition, and compound 59 was included as a negative control for P-gp inhibition. N = 9, with three samples per trial, three trials. Statistical significance determined using GraphPad Prism, Student’s T test of the mean. Significance was determined using a Student’s T test of the mean; P > 0.05 = N.S., P < 0.05 = *, P < 0.01 = **, P < 0.001 = ***, P < 0.0001 = ****.
2.3.8. Post-Testing Screens with SWISS-ADME Reveal the Inherent Difficulties in Predicting Transport Substrates of P-gp

The top hits from our cell-based assays were submitted for a post-hoc analysis via the SWISS-ADME server [53]. Notably, almost all of our compounds (except for 96, 97, 103 and 122) were predicted to be transport substrates of P-gp by SWISS-ADME (Table 2.3.4). These predictions conflict with the results of our LC-MS/MS assays, and suggest that further refinement of SWISS-ADME’s P-gp-substrate predictor is needed. Nonetheless, these data also underscore the inherent difficulty of predicting whether a particular compound will be a P-gp substrate. For our P-gp inhibitors that are also transport substrates, their average characteristics are as follows:

1. mean Molecular Weight (g/mol) of 464.5 ± 42.2,
2. mean Topological Polar Surface Area (TPSA, Å²) of 134 ± 21.6, and
3. mean LogP of 2.4 ± 1.4.

Our P-gp inhibitors that are *not* transport substrates tend to be slightly smaller and slightly more lipophilic, and to possess a slightly smaller TPSA:

1. mean Molecular Weight of 426.3 ± 50.4 g/mol,
2. mean TPSA of 109.5 ± 26.8 Å², and
3. mean LogP of 3.2 ± 1.1.

When comparing the average characteristics between the non-substrate and substrate inhibitors presented in Table 2.3.4, these differences were found to be statistically insignificant (Student’s T Test, GraphPad Prism). It is worth noting that the solubility predictions made by SWISS-ADME for each compound were generally correct.
<table>
<thead>
<tr>
<th>SMU#</th>
<th>MW (g/mol)</th>
<th>TPSA (Å²)</th>
<th>Consensus Log P_{oct/wat}</th>
<th>Consensus Log S</th>
<th>Correct Solubility Prediction?</th>
<th>Predicted P-gp Substrate</th>
<th>Correct P-gp Substrate Prediction?</th>
<th>MolPort ID</th>
</tr>
</thead>
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<tr>
<td>59</td>
<td>323</td>
<td>189</td>
<td>-0.59</td>
<td>Soluble</td>
<td>✓</td>
<td>Yes</td>
<td>ND</td>
<td>002-662-098</td>
</tr>
<tr>
<td>60</td>
<td>522</td>
<td>146</td>
<td>3.88</td>
<td>Poorly</td>
<td>×</td>
<td>Yes</td>
<td>×</td>
<td>000-779-897</td>
</tr>
<tr>
<td>61</td>
<td>480</td>
<td>164</td>
<td>0.3</td>
<td>Soluble</td>
<td>×</td>
<td>Yes</td>
<td>✓</td>
<td>001-984-582</td>
</tr>
<tr>
<td>66</td>
<td>411</td>
<td>121</td>
<td>1.74</td>
<td>Soluble</td>
<td>✓</td>
<td>Yes</td>
<td>×</td>
<td>005-923-195</td>
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<tr>
<td>70</td>
<td>430</td>
<td>120</td>
<td>3.23</td>
<td>Poorly</td>
<td>✓</td>
<td>Yes</td>
<td>×</td>
<td>005-927-826</td>
</tr>
<tr>
<td>71</td>
<td>455</td>
<td>104</td>
<td>3.74</td>
<td>Poorly</td>
<td>✓</td>
<td>Yes</td>
<td>×</td>
<td>005-926-380</td>
</tr>
<tr>
<td>78</td>
<td>425</td>
<td>113</td>
<td>3.28</td>
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<td>×</td>
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<td>89</td>
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<tr>
<td>96</td>
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<td>94</td>
<td>4.49</td>
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<td>×</td>
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<td>027-713-270</td>
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<tr>
<td>97</td>
<td>427</td>
<td>81</td>
<td>3.8</td>
<td>Moderate</td>
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<td>No</td>
<td>✓</td>
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<tr>
<td>101</td>
<td>494</td>
<td>88</td>
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<td>×</td>
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</tr>
<tr>
<td>103</td>
<td>490</td>
<td>111</td>
<td>4.11</td>
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<td>×</td>
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<td>×</td>
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<tr>
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<td>100</td>
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<td>×</td>
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<tr>
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<td>✓</td>
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<tr>
<td>124</td>
<td>392</td>
<td>116</td>
<td>2.38</td>
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<td>No</td>
<td>×</td>
<td>005-930-433</td>
</tr>
</tbody>
</table>

Table 2.3.4. **Computational Analysis of Compounds and Comparison to In Vitro Data.**

The top performing compounds from computational screens and *in vitro* assays were subjected to retroactive analysis with SWISS-ADME. Compounds 59 and 89 were included as examples of compounds with significantly different properties that did not reverse MDR; 59 and 89 were used as internal negative controls for P-gp inhibition with the A2780-ADR and A2780 cell lines. The predicted aqueous solubility was compared to that observed in the laboratory. The predicted classification as a P-gp substrate was compared to the LC-MS/MS intracellular accumulation data. Other characteristics are as described in Table 2.2.1. ND = no data. Compounds shaded grey are the top hits from *in vitro* assays that 1) reverse MDR, 2) do not outperform PTX alone in chemo-sensitive cancer cells, 3) are non-toxic to noncancerous cells, and 4) are not P-gp substrates.

### 2.4. Discussion

It has been suggested that the hit rate for blind high throughput screening (HTS) against proteins with no known target location is between 0.25-1% [97]. For identification of lead compounds effective at < 100 μM concentrations, the median hit rate of Virtual Screening-assisted efforts (VS) is approximately 13% [97]. 103 studies reported hit rates greater than 25%; however, 75% of these studies tested fewer than 20 compounds and used a sub-100 μM cutoff for
effective concentration (21). These trends of small sample size and incredibly high cutoff concentrations have been observed in other virtual-assisted studies [98-100].

Additionally, many virtual-assisted studies have the benefit of high-resolution structures of the protein target, which frequently include co-crystallizations with known inhibitors. We note that suitable high-resolution structures of human P-gp were unavailable when these screens were performed; thus our screens were performed with our homology model of human P-gp in dynamic conformations (Figure 3.2.3) [25, 64]. Regardless, the success of the screening methodology presented here serves as significant validation of 1) our dynamic P-gp model, and 2) our subtractive docking techniques. By targeting the NBDs and not the DBDs, we successfully identified several inhibitors of P-gp that are not transport substrates of the pump itself (Figure 2.3.2). Furthermore, if these inhibitors are proven to interact with the NBDs of P-gp in subsequent biophysical assays, then they could form the basis for a 4th generation of P-gp inhibitors (Table 2.2.1). Indeed, we have identified – and optimized – members of this putative 4th generation in our initial screens that targeted the NBDs [3-6]. The structures of our novel P-gp inhibitors are shown in Figure 2.4.1.
The molecular structures of compounds 70, 78, 96, 97, 101, 103, 111, 122, and 124 were downloaded from MolPort (www.molport.com). The unique MolPort ID (###-###-###) of each compound is shown below the compound’s unique identifier (SMU#). Selected chemical characteristics of these compounds are shown in Table 2.3.4.

The ABC transporter superfamily contains a multitude of clinically relevant protein targets that exhibit similar conformational dynamics to P-gp [1, 8]. With recent advances in cryo-EM, high-resolution structures of these transporters are increasingly available for use in virtual screening efforts. We feel that the methodology presented in this work, particularly the use of subtractive docking methods, could be applied to drug screens targeting many members of the ABC transporter family.
2.5. Conclusion

Using multiple receptors, applied MD simulations, and predicted chemical and toxicity properties, this work presents an enhancement of traditional computational screening methods by a significant factor. We report a 13% global hit rate with a < 15 μM cutoff – a significant improvement compared to other top virtual-assisted drug discovery methods. Of the 67 compounds identified by *in silico* screens against dynamic P-gp targets, 15 compounds significantly re-sensitized the MDR P-gp overexpressing DU145-TXR cancer cells to PTX. Of these top hits, 9 significantly re-sensitized the P-gp overexpressing A2780-ADR line to PTX, and thus are not specific to the DU145-TXR cell line. All 9 of these inhibitors were also observed to be non-toxic to non-cancerous HFL1 cells. Lastly, 6 out of 9 of these inhibitors were not observed to be transport substrates of the pump itself. We have successfully identified 6 candidates for lead optimization efforts – compounds 70, 78, 96, 97, 101, and 111.

2.6. Methods

2.6.1. Receptor Generation for Virtual Screens against Human P-gp

A putative catalytic cycle of P-glycoprotein (P-gp, *ABCB1*) was simulated using Targeted Molecular Dynamics (TMD) simulations with NAMD as described in [25, 26]. From these TMD simulations, we produced ten distinct conformations of P-gp: an approximate 4KSB position, 3B5X position, two 3B5Z positions, two 2HYD positions, and two positions between 4KSB and 3B5X named the “Transition” position (Figure 2.3.2). Three structures (4KSB_DB, Transition_DB, and 3B5X_DB) (Figure 2.3.2, Boxes 1-3) were used for docking against the drug binding domains (DBD), and five conformations (3B5Z_NBD_1, 3B5Z_NBD_2, 2HYD_NBD_1, 2HYD_NBD_2 and Transition_NBD) were used for docking against the nucleotide binding domains (NBD) (Figure 2.3.2, Boxes A-D). The NBD search areas shown in
Figure 2.3.2 were divided into two separate dock boxes, each targeting the respective individual NBD. Receptor files were prepared as PDBQTs for docking using AutoDock Tools [59].

2.6.2. Ligand Selection and Preparation for Docking

Compounds were taken from the ZINC12 Clean Drug-Like Subset of 13,195,609 molecules (31). Molinspiration (mib) software was used to calculate logP, polar surface area, molecular weight, number of hydrogen-bond donors and acceptors and number of rotatable bonds for each ligand [101]. To predict the aqueous solubility of our experimental compounds, the AMSOL program was used to calculate polar and apolar desolvation energies [102]. The criteria for ligand selection conformed to Lipinski’s rule of 5: (1) a molecular weight less than 500 and greater than 150 Daltons; (2) an octanol-water partition coefficient (log P) less than 5; (3) a topological polar surface area less than 150 Å²; (4) less than 5 hydrogen donors; and (5) less than 10 hydrogen acceptors.

The dataset used in the purchase of compounds SMU58-68 was obtained from a precalculated 90% Tanimoto cutoff set containing 123,510 “Clean Drug-Like Compounds” from the Zinc¹² database [103]. Ligands were obtained in mol2 file format at a pH of 7. We found that this subset was not structurally diverse, especially in molecular weight, and therefore only 10 compounds were purchased from it. For compounds SMU 68-124, we generated a custom 95% Tanimoto set from the ZINC Clean Drug-Like Subset using cactvs (http://www.xemistry.com/) and SUBSET (https://cactus.nci.nih.gov/subset/); this resulted in a final dataset of 158,000 diverse molecules. The mol2 files from the ZINC database corresponding to the 158,000 compounds were converted to PDBQT format using Open Babel [61]. Open Babel 2.3.2 was used for the Zinc¹² 90% Tanimoto dataset and Open Babel 2.4.0 was used for the custom 95% Tanimoto dataset.
2.6.3. **Ligand Docking and Iterative Screening with Autodock Vina**

AutoDock Vina was used to dock each of the 158,000 molecules against each target shown with an exhaustiveness of 128 (default exhaustiveness is set at 8), totaling over 4.2 million individual docking experiments [39]. The resultant ligand docking poses were ranked by predicted affinities/binding energies. All docking calculations were performed on the High-Performance Computing Facilities at SMU using the ManeFrame computing cluster. Docking results were downloaded to a Linux workstation, where bash scripts were used to extract and organize the individual files. For each ligand-target combination, the docking pose with the best predicted binding energy (kcal/mol) was extracted and used to calculate an estimated equilibrium constant of dissociation (estimated $K_d$). For concise evaluation of each ligand, the binding energy for each receptor-ligand pair was combined with the ligand molecular weight, logP, apolar desolvation (kcal/mol), polar desolvation (kcal/mol), H-bond donors, H-bond acceptors, net charge, topological polar surface area (TPSA), rotatable bonds, and simplified molecular-input line-entry system (SMILES string). The R language for statistical computing and graphics was used for manipulation and analysis of the combined data. To identify ligands that might preferentially bind to the NBDs of P-gp instead of the DBDs, we looked for molecules with a high estimated $K_d$ NBD : estimated $K_d$ DBD ratio.

The PDBQT output files from Autodock Vina were converted into PDB file format compatible with Visual Molecular Dynamics (VMD), a PNG picture of the ligand was generated, and the log files of the ligand docking were organized based on the receptor [39, 56]. The top 100-500 ligands with the best ratio of DBD/NBD (i.e., preferential docking to the nucleotide binding domains versus the drug binding domain) were used in searches of the ZINC database to
return molecules with 70-99% similarity in structure. This produced a second dataset of ~100 - 10,000 molecules per top ligand which was docked and analyzed in the same way as the first set.

2.6.4. Property Prediction and Selection

The top 100 ligands against each target were subjected to property prediction using the Online chemical database (OCHEM) [104]. Compounds that passed certain filtering requirements (e.g., good ratio of binding to nucleotide binding domain over drug binding domain affinity) were loaded into OCHEM as a single SDF (structure-data file) containing all molecules as SMILES (simplified molecular-input line-entry system) strings. OCHEM processing of ligand SMILES produced predictions for LogP and Solubility, Environmental toxicity, Ames test, CYP3A4 inhibition, CYP2D6 inhibition, CYP2C19 inhibition, CYP2C9 inhibition, CYP1A2 inhibition, Melting Point best (Estate), Pyrolysis O Estate submodel, Water solubility (GSE) based on logP and Melting Point, ALOGPS 2.1 logS, ALOGPS 2.1 logP, DMSO solubility, and any known toxic effects from literature. Along with the specific docking location to the receptor as visualized in VMD, the structural, chemical, and docking information was used to select ligands for purchase and subsequent testing.

QSAR properties are based upon the OCHEM database set of [104]. The AMES test for biotoxicity was virtually performed using the OCHEM Database. This virtual AMES test employs the Sushko 2010 mutagenicity metrics and reports an accuracy of 30-60% based upon a training set of 4361 compounds [104]. Compounds were also virtually assessed for predicted interactions with a suite of Cytochrome P450 enzymes via OCHEM. Using this method, compounds were labeled as “active”, i.e., predicted to interact with the respective enzyme, or “inactive, i.e., not predicted to interact with the respective enzyme. While the accuracy of the predictive abilities of the CYP3A4, CYP2D6, CYP2C19, CYP2C0 and CYP1A2 computational
BioAssays posted to PubChem are not provided by OCHEM, their methods of analysis were calculated using the same methods as the AMES test. The Cytochrome P450 screens from OCHEM do report a confidence interval for the interaction predictions, and reports with a confidence below 0.60 were not considered, i.e., a compound prediction of CYP3A4 inhibition with confidence of 0.20 would be treated as if there was no result for that field.

2.6.5. Testing of Experimental Compounds in Cell-Based Assays

2.6.5.1. Cell Lines and Cell Culture

The chemotherapeutic sensitive DU145 human prostate cancer cells and the multidrug resistant sub-line, DU145-TXR, were generous gifts from Dr. Evan Keller (University of Michigan, Ann Arbor, MI). The MDR, P-gp overexpressing DU145-TXR cells were maintained under positive selection pressure by supplementing complete medium with 10 nM paclitaxel (Acros Organics, NJ). In addition to the cell lines, the chemotherapeutic sensitive A2780 ovarian cancer cells (93112519, Sigma) and the multidrug resistant A2780-ADR (93112520, Sigma) were maintained in complete RPMI media consisting of RPMI-1640 with L-glutamine, 10% fetal bovine serum (FBS; Corning), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C and 5% CO2. The MDR, P-gp overexpressing A2780-ADR cell line was maintained under positive selection pressure by supplementing complete medium with 100 nM Doxorubicin (Fisher Scientific, NJ). The non-cancerous HFL1 cell line was maintained in complete F12K media consisting of F12K with 10% fetal bovine serum, 100 U/mL penicillin, and 100 ug/mL streptomycin (F12K source). Flasks and 96 well plates that were used with the non-cancerous line HFL1 were pre-treated with 0.01 mg/ml Collagen Type 1 and rinsed with PBS.
2.6.5.2. MTT Viability Assays

The MTT assay measures the reduction of the yellow, water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to blue, insoluble formazan crystals by cellular reductase processes in living cells [95]. Cancerous cell lines were seeded at 3,000 cells per well in a 96 well plate and incubated for 48 hours; the non-cancerous HFL1 line was seeded at 4,000 cells per well. Cells were then treated with experimental compounds for 48 hours. At 48 hours, MTT solution was added (20 µL/well of 5 mg/mL MTT in PBS). After incubation for 4 hours, the media was removed, and the formazan crystals were dissolved in 100 µL DMSO per well. Plates containing the semi-adherent A2780 or A2780-ADR lines were spun at 1400 rpm for 3 minutes prior to aspiration of media and the addition of DMSO. Plates were shaken for 10 minutes at 500 rpm on an Orbital shaker. The absorbance was measured at 570 nm using a Bio-Tek Cyation 5 (Bio-Tek, Winooski, VT). The measured absorbance value per well correlates with the number of metabolically active cells in that well [95]. The percent of cell viability was calculated using the following equation:

\[
\text{% Viability} = 100 \times \frac{\text{Fluorescence}_{\text{experimental cells}} - \text{Fluorescence}_{\text{background}}}{\text{Fluorescence}_{\text{DMSO-treated cells}} - \text{Fluorescence}_{\text{background}}} 
\]

For DU145-TXR, DU145, and A2780 cells - data are the mean ± one standard deviation, n = 8 samples per compound, from at least two independent trials per compound. For A2780-ADR cells, data are the mean ± one standard deviation with n = 12 samples per compound, from at least two independent trials per compound. The semi-adherent A2780-ADR cells exhibited a much higher variability in MTT assays; thus the larger sample size was used for this cell line.
2.6.5.3. Resazurin Viability Assays

Resazurin viability assays were performed as described in [5] by Courtney Follit. Cells were trypsinized from monolayers and seeded in 150 µL of complete RPMI medium in 96 well plates and incubated for 24 hours at 37 °C in a humidified incubator (5% CO₂). Cells were then treated with 15 µM of experimental compound in the presence or absence of 500 nM PTX. Data are presented as the mean of two separate experiments, n = 6 samples per compound, from two independent trials.

2.6.5.4. Cell Culture Assays for LC-MS/MS Quantification

DU145-TXR cells were passaged (as described in Section 2.6.5.1), strained 5x using a sterile cell strainer (Fisherbrand, sterile, polypropylene, 40 µm mesh), and seeded in 6-well plates (Greiner, sterile, polystyrene). Each well was seeded with 1 mL of DU145-TXR cell suspension at 350,000 cells / mL; 2 mL of fresh complete medium was then added to each well, bringing the total volume to 3 mL. Plates were incubated for 48 hours at 37°C in a humidified incubator, as described in Section 2.6.5.1.

After 48 hours, the media was replaced with 1.9 mL (per well) of fresh complete RPMI media. Using two 50 µL treatments, cells were treated with one of the following combinations:

A. 50 µL of Compound/DAU solution and 50 µL of 2 % DMSO media
   - final [Compound/DAU] = 5 µM in well; or

B. 50 µL of Compound/DAU solution and 50 µL of TQR solution
   - final [Compound/DAU] = 5 µM in well, final [TQR] = 500 nM in well.

The full calculations and makeup of these solutions are shown in Table 2.6.2. Our experimental compounds and TQR are dissolved in DMSO; thus, a vehicle control solution – 2%
DMSO Complete RPMI media – was prepared for use in the “compound alone” wells, to maintain a consistent concentration of DMSO in all samples. Dosing was performed using an electric multichannel pipette. For an example of an experimental setup, please see Table 2.6.1 below.
Table 2.6.1. **Example Plate Setup – Single LC-MS/MS Trial.**

Here we show an example setup of a typical LC-MS/MS experiment performed with DU145-TXR cells. A single trial of an experimental compound uses an entire 6-well plate, and includes three samples treated with 5 μM compound alone, and 3 samples treated with 5 μM compound and 500 nM Tariquidar (TQR). **Daunorubicin** – Here we show how a single sample pair – 5 μM DAU alone and 5 μM DAU + 500 nM TQR, was included in each LC-MS/MS experiment as a qualitative positive control. The 5 μM DAU + 500 nM TQR sample turns visibly red to the naked eye. These results were documented in L. Ammerman’s lab notebook each time. To supplement these qualitative positive controls, a full Daunorubicin sample was prepared as shown in Plate 1 (substitute “70” with “DAU”) and analyzed via LC-MS/MS. **Blank** – these wells were left empty.

<table>
<thead>
<tr>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Plate 3</th>
</tr>
</thead>
<tbody>
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<td>5 μM 70</td>
<td>5 μM 70 + 500 nM TQR</td>
<td>5 μM 96</td>
</tr>
<tr>
<td>5 μM 70</td>
<td>5 μM 70 + 500 nM TQR</td>
<td>5 μM 96 + 500 nM TQR</td>
</tr>
<tr>
<td>5 μM 70</td>
<td>5 μM 70 + 500 nM TQR</td>
<td>5 μM 96 + 500 nM TQR</td>
</tr>
<tr>
<td>blank</td>
<td>blank</td>
<td>blank</td>
</tr>
<tr>
<td>Compound</td>
<td>Equation</td>
<td>Volume TQR</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Tariquidar</td>
<td>$(1 \times 10^{-3} \text{ M TQR})(V_i) = (1000 \mu L)(5 \times 10^{-7} \text{ M TQR}) \left(\frac{2000 \mu L_{\text{final}}}{50 \mu L_{\text{added}}}\right)$</td>
<td>$V_i = 20 \mu L$ of 1 mM TQR in DMSO</td>
</tr>
<tr>
<td>Compound</td>
<td>$(1 \times 10^{-2} \text{ M Compound})(V_i) = (400 \mu L)(5 \times 10^{-6} \text{ M Compound}) \left(\frac{2000 \mu L}{50 \mu L}\right)$</td>
<td>$V_i = 8 \mu L$ of 10 mM Compound in DMSO</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>$(5 \times 10^{-2} \text{ M DAU})(V_i) = (400 \mu L)(5 \times 10^{-6} \text{ M DAU}) \left(\frac{2000 \mu L}{50 \mu L}\right)$</td>
<td>$V_i = 1.6 \mu L$ of 50 mM DAU in DMSO</td>
</tr>
<tr>
<td>DMSO control</td>
<td>Equation</td>
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</tr>
<tr>
<td>solution</td>
<td>$(5 \times 10^{-2} \text{ M DAU})(V_i) = (400 \mu L)(5 \times 10^{-6} \text{ M DAU}) \left(\frac{2000 \mu L}{50 \mu L}\right)$</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6.2. **Solution Preparation for an LC-MS/MS Experiment.**
Solutions were prepared at room temperature (~27 °C) in sterile 2mL Eppendorf microcentrifuge tubes in a cell culture hood. Experimental compounds were purchased from Mol-Port (https://www.molport.com/shop/index) and dissolved in DMSO to create 10 mM stocks (Alfa Aesar, cell culture grade). Daunorubicin (Fisher Scientific, NJ) was prepared from 50 mM stocks dissolved in DMSO. Complete RPMI media was prepared as described in Section 2.6.5.1.
LC-MS/MS experiments were performed in triplicate with two independent trials per compound, n = 6 total, or three independent trials per compound, n = 9 total. As shown in Table 2.6.1, the P-gp substrate, Daunorubicin (DAU), was included in each individual trial as a qualitative positive control. We observed that the TQR-inhibited sample with DAU turns visibly red to the naked eye. In addition to these internal qualitative controls performed with each experiment, a full sample of DAU-treated cells was prepared as well for analysis and quantification, with n = 6 total, two independent trials, using the setup shown in Table 2.6.1.

Cells were incubated for 2.5 hours at 37 °C in a humidified incubator. After incubation, cells were trypsinized using ice-cold 0.05% Trypsin-EDTA (Gibco) at 37 °C for 3 minutes. Plates were then removed from the incubator and placed on ice until use. Each well was scraped with a cell scraper to aid detachment of cells under cold conditions, and the resultant cell slurry was aspirated and stored in a sterile 15 mL falcon tube. Samples were then spun at 2400 rpm at 4 °C, after which media was carefully removed with a glass aspirator. The cell pellet was washed by adding 2 mL of ice-cold Hank’s Balanced Salt Solution (HBSS, Gibco) and vortexed. Samples were spun once again at 2400 rpm and at 4 °C. The supernatant was carefully removed with a glass aspirator to avoid disturbing the pellet. The final cell lysates were diluted in 600 µL of ice-cold HBSS, flash-frozen in liquid nitrogen, and stored at -80°C until analysis. An overview sample harvesting and processing is shown in Figure 2.6.1.
2.6.5.5. Relative Quantification in LC-MS/MS Intracellular Accumulation Assays

LC-MS/MS intracellular accumulation assays were performed in collaboration with the Pharmacology Core at the University of Texas Southwestern Medical Center (UTSW). LC-MS/MS assays were performed as described in [5] with the following modification – instead of calculating a quantitative assessment of the absolute amount of compound in each sample (as was done in [5]), a relative assessment was performed. This method allowed us to quantify the
change – if any – in the relative amount of compound present in the samples when TQR was added. This quantification method can be described using the following equations:

\[
(2.2) \quad \text{Ratio Analyte / Internal Standard (IS)} = \frac{\text{Analyte Peak Area (counts)}}{\text{IS Peak Area (counts)}}
\]

\[
(2.3) \quad \text{Normalized Ratio} = \frac{\text{Ratio of Analyte / IS}}{\text{Sample Content (mg / ml)}}
\]

\[
(2.4) \quad \text{Mean Ratio} = \frac{\sum_{i=1}^{n} \text{Ratio}_i}{n \text{ samples}}
\]

In the preceding equations, “Analyte” refers to the experimental compound treatment, in this case referring to the experimental compound tested. The “Internal Standard” is a known substance, N-Benzylbenzamide, added in a constant amount to each sample. The “Peak Area”, expressed as “counts”, describes the area under the curve of the MS/MS spectra from the compound in question, and thus serves as a measure of that compound’s relative abundance. Thus, for each sample we express the relative abundance of experimental compound as a ratio of the Analyte abundance divided by the Internal Standard abundance (Equation (2.2). To account for any differences in the amount of cell lysate between samples, the ratio of each sample was normalized to its respective cell lysate content expressed in mg/mL, as shown in Equation (2.3). Lastly, as shown in Equation (2.4), we average the “Normalized Ratio” of the samples, performed in triplicate, to produce the final values shown in Table 2.3.1. These values allow a comparison of the relative abundance of compound between the “- TQR” and “+ TQR” samples, and to test for statistically significant differences. An example of these data is shown in Table 2.6.3.
<table>
<thead>
<tr>
<th></th>
<th>Analyte Peak Area (counts)</th>
<th>IS Peak Area (counts)</th>
<th>Analyte/IS</th>
<th>“Cell Content” mg/mL</th>
<th>Normalized Ratio Analyte/IS</th>
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<td></td>
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<td></td>
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<td><strong>Std. Dev.</strong></td>
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Table 2.6.3 Example LC-MS/MS Data for the Relative Quantification Method.
The Internal Standard used was N-Benzylbenzamide. “Cell Content” was measured as total content of cell lysate in mg/mL. Experimental peaks for each compound were determined using a sample of pure experimental compound at 10 mM dissolved in DMSO. These data are from one independent trial, with each treatment (compound alone, or compound and TQR) performed in triplicate. The Mean Normalized Ratio, along with its standard deviation, are used to calculate the statistical significance of any difference between the relative amounts of experimental compound.

2.6.5.6. Sample Preparation and Analysis with LC-MS/MS

LC-MS/MS quantification was performed as follows. Cell lysate was aliquoted into Eppendorf tubes. In contrast to [5], blank cell lysates were not spiked with varying concentrations of each compound to create a standard curve, since compound amounts were normalized to abundance of the internal standard. Instead, after the initial aliquot step, MeOH containing 50 ng/mL N-Benzylbenzamide was added to each sample. Samples were vortexed briefly, incubated at 27 °C for 10 minutes, and spun at 13,200 RPM for 5 minutes. The
supernatant was transferred to a new Eppendorf tube, and spun once more at 13,200 RPM for 5 minutes. The supernatant was then transferred to an HPCL vial, and subsequently analyzed using LC-MS/MS using a Sciex 4000QTRAP mass spectrometer coupled to a Shimadzu Prominence LC.

Chromatography conditions were as follow - Buffer A contained of water + 0.1% formic acid, and Buffer B contained of methanol + 0.1% formic acid. The column flow rate was 1.5 mL/min using an Agilent C18 XDB, 5 micron packing 50 × 4.6 mm column. After the addition of the organic solvent, the remaining cell lysate was resuspended in 0.1 M NaOH, boiled for 5 min, and mixed with 1:50 B:A reagent (Thermofisher BCA Kit) to determine the cell lysate concentration in each sample. A BSA standard curve was then prepared in water and mixed using the same ratio. The samples were incubated 30 min at 37 °C and absorbance was read at 562 nm. The ratio of Analyte to Internal Standard was then normalized to the lysate content for each individual sample. The data for each trial are shown in Table 2.3.1. The means, standard deviations, and resultant statistical significance are shown in Table 2.3.2. Statistical significance was determined using a Student’s T Test in GraphPad Prism.

2.6.5.7. Daunorubicin Accumulation Assays

These assays are based upon those reported in [5]. DU145-TXR cells were seeded in 96 well plates in complete RPMI medium at 15,000 cells per well. Cells were allowed to grow for 48 hours in a humidified incubator at 37 °C. After 48 hours, media was refreshed and replaced with fresh complete medium. Cells were treated with 10 µM of experimental compounds in the presence or absence of 10 µM Daunorubicin (DAU). After a 2 hour incubation at 37 °C, media was removed and cells were washed twice with cold Phosphate Buffered Saline (PBS) solution.
Cells were lysed in PBS containing 0.5% SDS and 0.5% Triton X-100 and shaken on an Orbital Shaker for 10 min at 500 rpm. DAU fluorescence was read using the Cytation5 plate reader, excitation/emission 488 nm / 575 nm. Data are presented as the mean ± one standard deviation from three independent experiments, n = 3 samples per experiment, n = 9 total. Statistical significance was determined with a Student’s T test in GraphPad prism. None of the experimental compounds exhibited significant background fluorescence at the tested excitation and emission range.

\[
\Delta DAU \text{ Fluorescence} = \frac{\text{Fluorescence of (Compound + DAU) treated cells}}{\text{Fluorescence of DAU treated cells}}
\]
### Supplemental Table 2.6.6 Testing of Compounds Against DU145-TXR Cells with Resazurin Assays.

Data provided, and testing performed, by Courtney Follit. Cells were incubated with 15 µM compound with or without 500 nM PTX for 48 hrs; survival was subsequently determined with the Resazurin viability assay. Data represent the mean of two separate experiments performed in triplicate. Column 4 is the difference between the means presented in Columns 2 and 3.

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CHAPTER 3.

These data published – PLOS One, McCormick & Ammerman et al. 2021.

3.1, Introduction

Alzheimer’s disease (AD) is a progressive and irreversible neurodegenerative disease that primarily affects geriatric populations and accounts for approximately 80% of all dementia diagnoses [72]. One of the pathological hallmarks of AD is deposition and accumulation of amyloid-β (Aβ) in the brain, which is thought to be caused not only by increased Aβ production, but also by decreased clearance of Aβ from the brain [105-108]. Although the ‘Amyloid hypothesis’ regarding AD has been hotly debated, the best available evidence suggests that an imbalance between the production and clearance of Aβ peptides contributes to the onset and progression of AD [108]. The intricate relationship between Aβ peptides and the onset of AD is beyond the scope of this work; thus, we sought to explore one of the outstanding complexities of Aβ clearance from the brain: the efflux of Aβ peptides by P-glycoprotein across the Blood Brain Barrier (BBB).

There is compelling preclinical evidence that Aβ efflux across the BBB is a multistep process involving several cofactors, with P-gp as a critical component Figure 3.1.1 [72, 108-115]. First, Apolipoprotein E (ApoE) and Aβ associate with LDL Receptor Related Protein 1 (LRP-1) at the abluminal membrane of the BBB. LRP-1 mediates Aβ uptake across the abluminal membrane of the brain capillary endothelial cell [72, 113, 116]. Assisted by PICALM and Rab11, Aβ and LRP-1 associate with P-gp intracellularly in sorting endosomes [111, 113].
The exact molecular mechanisms of these endosomal interactions are still an open question. Lastly, Aβ is actively exported by P-gp across the luminal membrane and into the blood [72, 113, 116, 117]. Notably, a recent study by Hartz et al. demonstrates that this pathway is perturbed in mouse models of AD – exposure to Aβ can trigger the ubiquitination, internalization, and degradation of P-gp (Figure 3.1.1).

P-glycoprotein is thought to transport both Aβ(1-42) and Aβ(1-40) (Aβ42 and Aβ40, respectively). Aβ40 is the most common isoform in the brain; however, in certain forms of AD, the 42 residue isoform Aβ(1-42) has also been shown to accumulate significantly in the brain [118]. Aβ(1-42) is also regarded as the more fibrillogenic of the peptides produced from amyloid precursor protein (APP) degradation [118-120]. Both Aβ-42 (4514.04 Da) and Aβ-40 (3429.80 Da) are significantly larger than the largest known substrate of P-gp, cyclosporine A (1,202.61 Da) [79, 121, 122].
Figure 3.1.1 *Efflux of Aβ across the Blood Brain Barrier is a Multistep Process.* Studies suggest that clearance of Aβ is a multistep process mediated by several protein factors. **Step 1**) Aβ may associate with Apolipoprotein E (ApoE), which is a ligand of LRP-1 [113, 123]. LRP-1 is expressed on the abluminal surface of the BBB and mediates uptake of Aβ across the abluminal membrane. **Step 2**) LRP-1, Aβ and P-gp associate intracellularly in endosomes with assistance from PICALM and Rab11 [111, 113]. The exact molecular mechanisms of association are unclear. **Step 3**) P-gp mediates efflux of Aβ across the luminal membrane and into the blood [115, 124, 125]. *AD*) P-gp is downregulated in AD brains, and this is thought to occur by an Aβ-triggered ubiquitination, internalization, and proteasomal degradation of the transporter [126]. Figure created by LA using the Biorender program (www.biorender.com).

As discussed in CHAPTER 2, P-glycoprotein is a member of the ATP Binding Cassette (ABC) transporter family and effluxes a variety of substrates [124]. In particular, studies involving mouse models of AD support an active role for P-gp in the transport of AD-associated Aβ [112, 113, 124, 125, 127-129]. For example, the expression of P-gp in brain capillaries is inversely correlated with deposition of Aβ in the brain [72, 130]. Restoring P-gp expression at the BBB reduces the accumulation of Aβ in the brain [125]. Furthermore, P-gp is downregulated
in AD brains by an Aβ-triggered process of P-gp ubiquitination, internalization, and proteasomal degradation (Figure 3.1.1) [126]. In humans, these studies are supported by comparing cognitively normal brains to age-matched brains of AD patients; the brains of AD patients exhibit significant decreases in P-gp expression and significant increases in Aβ deposition [128, 129]. Additionally, endothelial BBB expression of P-gp declines as humans age, and this decrease in P-gp expression is accompanied by reduced functioning of the BBB [128, 131, 132]. Taken together, these data suggest an active role of P-gp in Aβ clearance from the brain.

However, in vitro studies of interactions between P-gp and Aβ have yielded mixed results. In 2001, Lam et al. observed that Aβ is transported by hamster P-gp [133]. Two subsequent studies, one using human colon adenocarcinoma cells, the other using P-gp transfected porcine LLC cells, support these findings [124, 134]. Inhibition of P-gp in the human hCMEC/D3 cell line resulted in increased intracellular accumulation of Aβ40 [135]. A new study by Chai et al. provides compelling evidence that both Aβ40 and Aβ42 interact with and are transported by P-gp in vitro and ex vivo [115]. However, a study by Bello & Salerno using paired P-gp overexpressing and non-P-gp-overexpressing human carcinoma lines found that Aβ42 had no effect on the efflux of the P-gp substrate, pirarubicin [136]. This study also found that Aβ42 had no effect on the ATPase activity of P-gp in membrane vesicles [136]. Lastly, the overexpression of P-gp in polarized canine MDCK cells did not promote the transcytosis of radiolabeled Aβ40 in transwell assays [117].

In this study, we assessed the ability of P-gp to transport Aβ using both computational and in vitro techniques. Using explicit all atom MD simulations, we analyzed and modeled the transport mechanism of Aβ40 and Aβ42 by human P-gp. In biochemical assays, we showed that Aβ42 stimulates the ATPase activity of purified P-gp; however, this stimulation is dependent
upon the lipid environment used. In cell culture assays, we observed enhanced intracellular retention of fluorescently-labeled Aβ42 in the presence of Tariquidar, a potent P-gp inhibitor [137]. Our results indicate that Aβ peptides are transport substrates of P-gp, suggesting that P-gp may also be involved in the onset and progression of AD. Understanding the role of P-gp in AD may be of crucial importance for the development of future treatments, and may have implications for compounds targeting P-gp in cancer treatment.

3.2. Results

3.2.1. Targeted Molecular Dynamics Simulations Show Transport of Amyloid β by P-gp

To investigate whether P-gp is indeed capable of transporting the Aβ40 and Aβ42 peptides, we performed targeted molecular dynamics (TMD) simulations of Aβ-bound P-gp in a lipid bilayer. TMD simulations were performed using the techniques that were used by us to simulate P-gp-mediated transport in MD simulations [25, 64]. To generate a plausible starting point for our TMD simulations, three variants of AD-associated Aβ (Aβ40: PDB IDs 2LFM, 2M4J; Aβ42: PDB ID 1IYT) were docked to human P-gp in a putative starting conformation with the drug binding domains (DBDs) open to the cytoplasm [25, 39, 92, 138-140] (Figure 3.2.1 and Figure 3.2.3). We did not explore docking of Aβ peptides to the NBDs.
Figure 3.2.1 Aβ42 Docked with the DBDs of Human P-gp.
(A) The first frame of a representative simulation of Aβ42 (PDB 1IYT) bound to the drug binding domains of human P-gp. (B) The final frame of the same representative TMD simulation shown in (A). The N- and C-terminal halves of P-gp are colored turquoise or orange; Aβ42 is shown in purple surface representation; ATP and ADP are bound at the nucleotide binding domains and shown in van der Waals representation.

When docked to the DBDs, we found that each Aβ peptide occupied the previously identified R and H drug binding sites simultaneously, and that Aβ-DBD contacts were dominated by hydrophobic interactions (Figure 3.2.2, Table 3.2.1) [25, 141]. Our findings suggest that the Aβ peptides are indeed able to fit within the DBDs of P-gp despite their large sizes. These data are supported by a separate docking study of Aβ peptides performed by Callaghan et al, which used a conformation of P-gp similar to the 3B5X conformation shown in Figure 3.2.3 [142].
Despite the decrease in cavity volume relative to the 4KSB structure, the DBDs easily accommodated Aβ42 or Aβ40 [142].

Recent cryo-EM structures of human P-gp bound to the vincristine – a transport substrate – identified 13 residues within the DBDs that may be important for substrate binding and recognition [89]. Specifically, they are M68, M69, F983, Y310, I306, M949, E875, M986, Q946, Q347, F343, and Q990 [89]. In our docking studies of Aβ42 and Aβ40, the Aβ peptides interacted closely with 11 of the aforementioned residues, the exceptions being F983 and M68. Our docking studies support the hypothesis that these residues may be important for substrate binding and recognition.

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</tbody>
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Table 3.2.1. P-gp-Aβ Contacts During TMD Simulations Organized by Target.
Data are reported as the number and type of contacts made with the P-gp Drug Binding Domain at each stage of the simulation. Stages are as defined in Figure 3.2.3; contacts are defined as residues of the DBDs with an α-carbon within 3 Å of the simulated ligand. Residues are classified as Polar (SER, THR, CYS, ASN, GLN, TYR), Non-Polar (GLY, ALA, VAL, LEU, MET, ILE, PHE, PRO, TRP), positively Charged (LYS, ARG, HIS), negatively Charged (GLU, ASP).
Figure 3.2.2 **First and Final Frames of Representative TMD simulations.**
(A) Aβ40 (2LFM), (B) Aβ40 (2M4J), (C) Aβ42 (1IYT), (D) Polyarginine 42. Panels A-D are representative images of bound peptides at the start of molecular dynamics simulations, along with the chosen docking poses for each peptide. (A) 1IYT docked to the DBDs of P-gp with an estimated affinity of -7.2 kcal/mol. 1IYT is a solid state NMR structure of Aβ42 solved in an apolar microenvironment [138]. (B) 2LFM is a partially folded Aβ40 structure that was solved in an aqueous environment [140]. 2LFM docked to the drug binding domains (DBDs) of P-glycoprotein (P-gp) with a predicted affinity of -7.2 kcal/mol. (C) 2M4J is an Aβ40 fibril derived from brain tissue with Alzheimer’s disease [139]. 2M4J docked to the DBDs of P-gp with an estimated affinity of -7.1 kcal/mol. Panels E-H are representative images at the end of a TMD simulation. The Aβ peptides are shown in purple licorice representation or in surface representation colored for lipophilicity (teal = hydrophilic, gold = lipophilic, and white = neutral). The N- and C-terminal halves of P-gp are colored turquoise or orange.
As a negative control for our TMD simulations, we mutated every residue in the Aβ42 peptide (1IYT) into arginine, creating polyarginine 42 (P42). P42 was chosen because:

1. P42 does not share the traits of a typical transport substrate of P-gp [143].
2. P42 is similar in molecular weight and size to the tested Aβ peptides (Figure 3.2.2).
3. We were unable to identify a known, comparably sized non-substrate of P-gp.
4. Since P42 does not possess the characteristics of a typical P-gp substrate, P42 counters the concern that any molecule – known or disputed – would be transported as an artifact of the simulation.
5. In previous work using the same techniques, we have shown that a small, negatively charged molecule (methyl-pyrophosphate) is not transported by P-gp [25].

After assembly of a complete system (ligand, P-gp, lipids, ATP, ADP, water and ions) each system was relaxed in unbiased molecular dynamics (MD) simulations [25, 64]. TMD simulations were then performed as described in McCormick et al 2015 [25, 64]. Briefly, small forces were applied to α-carbons of P-gp to guide the protein through a series of conformational changes, thereby simulating a putative catalytic transport cycle (Figure 3.2.3). Except for the α-carbons of P-gp, external forces were not applied to the Aβ peptides, P42, or to any other atoms in the simulated systems. A total of 6 independent TMD simulations were performed per ligand.
Figure 3.2.3. **Structures of P-gp used as Targets for TMD Experiments.**
Here we show the sequence of target structures, in order, used for TMD experiments. Targets are labeled according to the PDB ID of their source structure. Targeted Molecular Dynamics (TMD) simulations were performed as described in McCormick et al. 2015 using a dynamic model of human P-glycoprotein and several low energy conformations of P-gp homologues [25, 64].
Figure 3.2.4. Movement of Aβ through the DBDs of P-gp in TMD Simulations. Simulations performed by James McCormick and Lauren Ammerman. The center of mass of (A) Aβ40 structure 2M4J, (B) Aβ40 structure 2LFM, (C) Aβ42 structure 1IYT, (D) Polyarginine 42 peptide and (E) Daunorubicin were calculated for each step of the simulated putative catalytic cycle of P-gp. Positional changes were calculated relative to the distance from starting coordinates of the ligand. Data represent the mean position of the center of mass (black line) ± one standard deviation from the mean shown in colored shading, n = 6 simulations per ligand. In these simulations, movement towards the cytoplasm is positive on the Z axis, and movement towards the extracellular space is negative. (F) shows the total mean distance traveled through the plane of the membrane (Z axis) ± one standard deviation. *Data for Daunorubicin is reproduced with permission from McCormick et al. 2015 [25]. Copyright 2015 American Chemical Society.
Figure 3.2.5. **Total Movement of Simulated Ligands in TMD Simulations.**
The center of mass of each ligand was calculated for each step of the simulations relative to the distance from the starting location in the X, Y, and Z axes. The plane of the membrane is parallel to the X and Y plane; movement through the membrane is oriented on the Z-axis. Distances are presented in Å. Six simulations were performed for each ligand; data represent the mean total distance ± one standard deviation from the mean. *Data for Daunorubicin is reproduced with permission from McCormick et al. 2015 [25]. Copyright 2015 American Chemical Society.

In each TMD simulation we observed vectorial movement of Aβ perpendicular to the membrane and towards the ‘extracellular space’ (Figure 3.2.4 and Figure 3.2.5). As the P-gp – bilayer system was oriented such that the membrane is parallel to the X – Y plane, movement through the membrane is expressed as movement along the Z-axis. In each set of simulations, we observed movement of the Aβ peptide from the cytoplasmic leaflet of the membrane to the extracellular leaflet of the membrane (Figure 3.2.4A-C).
In these simulations, the center of mass of Aβ40 (2LFM) moved 7.8 ± 1.3 Å from the cytoplasmic to the extracellular side of P-gp (Figure 3.2.4A); Aβ40 (from 2M4J) moved 9.4 ± 1.0 Å (Figure 3.2.4B); Aβ42 (from 1IYT) moved 8.4 ± 1.3 Å (Figure 3.2.4C). The data discussed here represent the mean movement across 6 independent simulations ± one standard deviation. The previously reported movement of the P-gp substrate daunorubicin (DAU) is shown in Figure 3.2.4E [25]. Daunorubicin moved an average of 10.0 ± 2.7 Å through the DBDs towards the extracellular space [25]. In summary, movement of the Aβ peptides through P-gp ranged from approximately 8 Å to 10 Å across all TMD simulations.

Both structures of Aβ40 (2LFM and 2M4J) were transported to the outer leaflet of the membrane in our simulations, but we observed a significant difference between the distance traveled by the two structures of Aβ40 (P = 0.04). When compared to the distance traveled by Aβ42, no significant difference was observed (Aβ40 2LFM vs. Aβ42, P = 0.43; Aβ40 2M4J vs. Aβ42, P = 0.17). Notably, despite the large discrepancy in size (DAU 527.5 g/mol, vs. 4514.04 Da Aβ42), no significant difference was observed between the movement of DAU and the Aβ monomers (Aβ42 vs. DAU, P = 0.20; Aβ40 2LFM vs. DAU, P = 0.09; Aβ40 2M4J vs. DAU, P = 0.56).

3.2.2. P-gp does not Transport Polyarginine 42 in TMD Simulations

Simulations with Polyarginine 42 (P42) were started at the initial docking pose of Aβ42 (1IYT) (Fig 1D). Figure 3.2.4D shows the average distance traveled by the center of mass of P42 during the transport cycle. In stark contrast to the behavior of the Aβ peptides, P42 was not moved through the membrane bilayer (n=6 independent simulations) but remained at a relatively stable position within the DBDs throughout each simulation (0.2 ± 2.6 Å, Figure 3.2.5). We observed a highly significant difference between the distance traveled by P42 and the distance
traveled by any of the Aβ monomers (P < 0.0001 for all three comparisons, respectively) (Figure 3.2.4F).

3.2.3. ATPase Activity of P-gp in the Presence of Aβ42

To test whether Aβ42 interacts directly with P-gp, Gang Chen used a series of in vitro ATPase assays of purified murine P-gp, which shares both 87% sequence identity and high functional similarity with human P-gp [144]. P-gp exhibits a relatively low rate of ATP hydrolysis in the absence of a transport substrate; the introduction of a transport substrate often results in a several fold increase in ATPase activity [3]. This comparison is not foolproof – as some non-substrates can stimulate the ATPase activity of P-gp [8, 145] – but these data are informative in combination with additional assays. Consequently, we used ATPase assays to assess whether monomeric Aβ42 modulates the ATPase activity of P-gp [3, 146]. We used purified murine P-gp in both mixed micelles and lipid bilayer nanodiscs; the latter are considered a more native-like lipid environment [147]. In these studies, 20μg of P-gp in micelles or 15μg of P-gp in nanodiscs were incubated with Aβ42 (molar ratio of 1:18) with or without 150 μM of verapamil (VPL), a substrate of P-gp.

In mixed micelles (Figure 3.2.6, blue bars), the ATPase activity of P-gp was stimulated by Verapamil (VPL) as expected. Similar to Bello & Salerno 2015, we found that Aβ42 alone did not stimulate the ATPase activity of P-gp in mixed micelles [136]. Furthermore, a combination of VPL and Aβ42 resulted in an increase of ATPase activity above that observed for VPL alone (P < 0.01). However, we did find that Aβ42 significantly stimulated the ATPase activity of P-gp in nanodiscs (Figure 3.2.6, orange bars, P < 0.005). With P-gp in nanodiscs, we observed a 1.42-fold basal increase in ATPase activity upon the addition of Aβ42. These findings closely agree with our contemporaries Chai et al., who observed a 1.5 fold-basal increase in
activity with P-gp in lipid vesicles upon the addition of 10 µM Aβ42 [115]. Interestingly, we observed that the combination of VPL and Aβ42 did not significantly stimulate ATPase activity relative to VPL alone. Our data suggest that the effect of Aβ42 on the ATPase activity of P-gp is dependent upon the membrane environment [8, 115, 145]. This could explain the contradictory findings of other ATPase activity studies of Aβ and P-gp, as many previous studies used membrane vesicles derived from a variety of cell types [124, 133, 136]. Our data also suggest that Aβ42 may interact directly with P-gp, supporting the hypothesis that Aβ42 is a putative transport substrate of P-gp.

Figure 3.2.6. The effect of Monomeric Aβ42 upon the ATPase Activity of P-gp. ATPase assays were performed by Gang Chen. The effect of monomeric Aβ42 upon the ATPase activity of P-gp was measured in both micelles and in lipid nanodiscs. The ATPase activities of each sample were normalized to the basal ATPase rate of P-gp in micelles (blue bars). Error bars represent one standard deviation from the mean. Significance tests were performed in GraphPad Prism using a Student’s T test for significance.
3.2.4. **P-gp does not transport Polyarginine 42 in TMD Simulations**

To test whether we could observe the results of transport of Aβ42 by P-gp in human cellular systems, a fluorescently labeled Aβ42 peptide (HiLyte488-Aβ42) was assayed for accumulation in the paired DU145 and DU145-TXR prostate carcinoma cell lines [94, 137, 148]. As mentioned in CHAPTER 2, the MDR DU145-TXR cancer cells greatly overexpress P-gp relative to the parental DU145 cells [94]. Additionally, in contrast to previous studies of Aβ and P-gp using paired cell lines, we used a strong, selective, and non-competitive P-gp inhibitor Tariquidar (TQR) to assess the accumulation of Aβ [124, 133, 136, 137]. Each cell line was treated with 1 µM HiLyte488-Aβ42, 1 µM of TQR, or a combination of 1 µM HiLyte488-Aβ42 and 1 µM TQR for 16 hours. The intracellular accumulation of HiLyte488-Aβ42 was imaged using confocal microscopy and quantified with ImageJ [149]; Figure 3.2.7C-N shows representative images of each treatment (n = 12 images per trial, two independent trials).
Figure 3.2.7 Intracellular Accumulation of HiLyte-488-Aβ42 in DU145 and DU145TXR Cells after P-gp Inhibition by Tariquidar.

The intracellular fluorescence of DU145 or DU145-TXR cells was measured by confocal microscopy after a 16 hour incubation with fluorescently labeled HiLyte-488-Aβ42. (A) Compared to DMSO-treated control cells (no added TQR, grey bar), DU145 cells showed a significant 19% (P < 0.0001) increase in mean intracellular fluorescence when inhibited by 1 µM Tariquidar (TQR; blue bar, top panel). (B) Compared to DMSO-treated cells, DU145-TXR cells showed a significant 24% (P < 0.0001) increase in mean intracellular fluorescence in the presence of 1 µM TQR (purple bar, bottom panel). Representative Images (C-E) show DU145 treated with 1 µM Aβ42 alone; (F-H) show DU145 treated with 1 µM Aβ42 and 1 µM TQR; (I-K) show DU145-TXR cells treated with 1 µM Aβ42 alone; (L-N) show DU145-TXR treated with 1 µM Aβ42 and 1 µM TQR.

In both the non-P-gp overexpressing and the P-gp overexpressing cell lines, we observed significant increases (P < 0.0001; P < 0.0001) in the accumulation of HiLyte488-Aβ42 in the
presence of TQR (Figure 3.2.7A-B and Figure 3.2.8). Although both cell lines showed a TQR-dependent increase in HiLyte488-Aβ42 accumulation, the P-gp overexpressing DU145-TXR cells exhibited the greatest increase in fluorescence (P < 0.0001) (Figure 3.2.8). Increased accumulation of HiLyte488-Aβ42 upon targeted inhibition of P-gp by TQR indicates that P-gp actively participates in the transport of HiLyte488-Aβ42 in human cellular systems.

Figure 3.2.8. **Fluorescence of HiLyte488-Aβ42-treated DU145 and DU145-TXR Cells.**
The intracellular fluorescence of paired chemotherapeutic sensitive/resistant cancer cell line (DU145 and DU145-TXR) was measured by confocal microscopy after incubation with 1 µM fluorescently labeled Aβ42 in the presence or absence of 1 µM Tariquidar (TQR). Statistical significance was determined using an unpaired T-test in Graphpad Prism; data are n = 24 images per treatment, two trials per treatment. Data are expressed as arbitrary units (a.u.) as calculated by the Integrated Density function of ImageJ [149-152].
3.3, Discussion

The mechanism by which P-gp might transport huge, flexible substrates – i.e., the Aβ peptides – remains unclear. Despite being particularly suited to exploring problems of this nature, MD simulations of Aβ and P-gp have not previously been performed. Thus, using our established TMD techniques, we explored how P-gp might transport Aβ40 and Aβ42 in TMD simulations. In each simulation we observed vectorial movement of Aβ through the P-gp DBDs and towards the extracellular space, with total movement ranging between 8 and 9.4 Å (Figure 3.2.4 and Figure 3.2.5). These distances correlate well with previously published movements of the P-gp substrate daunorubicin (DAU) in TMD simulations [25]. Interestingly, for both Aβ42 and the 2M4J structure of Aβ40, the bulk of observed movement occurred during the transition between the 3B5X and 2HYD conformations of P-gp, or when the DBDs switch from open-to-the-inside to open-to-the-outside (Table 3.3.1). For both DAU and the 2LFM structure of Aβ40, the bulk of observed movement occurred during the transition from 2HYD to 3B5Z, both of which are open-to-the-outside conformations (Table 3.3.1).

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<td>-9.4 ± 1.0</td>
</tr>
<tr>
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<td>-2.7 ± 1.9</td>
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<td>0.2 ± 2.6</td>
</tr>
<tr>
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<td>-5.5 ± 3.4</td>
<td>-4.6 ± 2.2</td>
<td>-10.1 ± 2.7</td>
</tr>
</tbody>
</table>

Table 3.3.1. Movement of Simulated Ligands According to Structural Transition.
Here we track the movement of each simulated transport ligand throughout the TMD simulation. Movements were calculated once P-gp reached the target structure defined in Figure 3.2.3. Movements are reported in Angstroms (Å) in the Z-coordinate axis and as the mean ± standard deviation, n = 6 per simulated ligand. Shaded columns show the change in movement between structures, if any.
Studies have shown that Aβ monomers can fold into structures with two β strands; these β strands allow the monomers to oligomerize and then to potentially assemble into AD-associated amyloid fibrils [153]. At the start of our simulations, the Aβ monomers were not in this folded conformation. It was, however, interesting to ask whether P-gp could somehow facilitate the folding of Aβ monomers during the transport process, and thus contribute to the formation of extracellular amyloid fibrils. In the folded state, both Aβ40 and Aβ42 stabilize the turn between β strands through a salt bridge between Asp23 and Lys28 [154]. In the simulations presented here, we report that this salt bridge was not observed to form for any significant amount of time (Figure 3.3.1).
Figure 3.3.1. **P-gp did not Contribute to the Folding of Aβ Monomers in TMD Simulations.**

The distance (Å) between the charged nitrogen (N) of LYS28 and each potentially charged oxygen (OD1, or OD2) of ASP23 in the respective Aβ peptides. Panels (A-C) show the position of K28 and D23 in each Aβ monomer. Graphs (D,E) show the mean distance (Å) between K28 and D23 in Aβ 42 (1IYT) during TMD simulations; graphs (F,G) show these data for Aβ 40 (2LFM); graphs (H,I) show these data for Aβ 40 (2M4J). Data represent the mean distance (Å) ± one standard deviation in shading (n = 6).
It is possible that hydrophobic interactions with the DBDs prevented the formation of any stable secondary structure by the Aβ peptides (Table 3.2.1). Indeed, residue contacts between the Aβ peptides and P-gp were dominated by hydrophobic, non-polar interactions throughout the transport process, with a notable increase in polar contacts as the DBDs opened to the extracellular space (Figure 3.3.2). We found that contacts between Aβ and charged residues of the DBDs contributed only a minority of the protein-ligand interactions. Our data suggest that transport by P-gp may not stabilize or contribute to the folding of Aβ monomers. Since Aβ monomers with a distinct folded structure were not simulated, the ability of P-gp to transport or disrupt folded Aβ monomers is unclear and warrants further study.
Figure 3.3.2. **P-gp-Aβ Contacts are Dominated by Hydrophobic Interactions.**

Data are reported as the number and type of residues in the P-gp Drug Binding Domain with an α-carbon within 3 Å of the respective Aβ peptide. Residues are classified as Polar (SER, THR, CYS, ASN, GLN, TYR), Non-Polar (GLY, ALA, VAL, LEU, MET, ILE, PHE, PRO, TRP), Positively Charged (LYS, ARG, HIS), or Negatively Charged (GLU, ASP). Residues were included in the total count, if the respective Aβ peptide interacted with the residue in 4/6 TMD simulations.

To date, studies of the effect of Aβ42 upon the ATPase activity of P-gp used membrane vesicles derived from different cellular systems [115, 133, 136]. Combined with our findings, these data support the growing consensus that the lipid environment strongly affects the behavior and transport activity of P-gp [77, 145, 155-158]. In our ATPase activity assays, we found that monomeric Aβ42 does not stimulate the ATPase activity of P-gp in micelles but does stimulate the ATPase activity of P-gp in nanodiscs (Figure 3.2.6). Therefore, we hypothesize that the
differences between our tested systems, and potentially the conflicting results of previous studies, may be due to interactions between P-gp and the different lipid environments. Indeed, a new study by Chai et al. shows that both Aβ42 and Aβ40 stimulate the ATPase activity of P-gp in lipid vesicles, providing further evidence that the Alzheimer’s associated Aβ peptides interact directly with P-gp, and may be transport substrates of P-gp [115].

Our ATPase studies also suggest that Aβ42 interacts directly with P-gp. However, at the tested concentrations, we observed that the Aβ42-stimulated ATPase activity was less than half of the VPL-simulated ATPase activity. This relationship may not extend across the entire possible concentration range. A possible explanation is that the large size of the Aβ42 peptide (4514 Da for Aβ42, versus 454.6 Da for VPL) may make it difficult for P-gp to move between structural states during the transport process. Aβ42 may dissociate slowly from the DBDs due to strong hydrophobic interactions and its large size, thus explaining the lower stimulation of ATPase activity compared to VPL (Figure 3.2.6). While increased stimulation of ATPase activity is considered a characteristic of P-gp substrates, it should be noted that some non-substrates can also stimulate the ATPase activity of P-gp [8, 145, 159].

To further test if P-gp can transport Aβ42, we performed fluorescence accumulation assays in a human cellular system. Our data show that inhibition of P-gp by TQR resulted in increased intracellular accumulation of fluorescently labeled Aβ42 (HiLyte488-Aβ42). To our surprise, we observed this increased accumulation in both P-gp overexpressing DU145-TXR cells and the parental, non-P-gp overexpressing DU145 cells (Figure 3.2.7).

Previous work by our group has shown that the chemotherapy-sensitive, non-P-gp-overexpressing DU145 cells do express detectable, but low, amounts of both P-gp and the Breast Cancer Resistance Protein (BCRP, ABCG2) [96]. While TQR is a strong inhibitor of P-gp,
Kannan et al. have shown that TQR can inhibit both P-gp and BCRP at concentrations greater than 100 nM [160]. However, the overexpression levels of P-gp and BCRP in DU145-TXR cells are significantly different – DU145-TXR cells overexpress P-gp much more than BCRP, relative to the parental DU145 cells [94, 96]. Quantitative One-Step qPCR assays reveal that the MDR DU145-TXR cancer cells overexpress P-gp by 14,000 fold compared to the PTX-sensitive DU145 parental line [96]. DU145 cells express very low – but detectable – amounts of P-gp [96], and are highly sensitive to PTX [94]. Furthermore, while recent evidence suggests that BCRP may also facilitate the transport of both Aβ40 and Aβ42, the great difference between the levels of BCRP and P-gp overexpression in DU145-TXR cells suggests that inhibition of BCRP is unlikely to significantly affect our results [161, 162].

The question remains – why did the uninhibited DU145-TXR cells exhibit significantly higher fluorescence than the uninhibited DU145 cells (Figure 3.2.8)? We hypothesize that this is due to reduced CD33 levels in the DU145-TXR cell line (3.75-fold decrease relative to DU145 cells), a byproduct of generating resistance through exposure to the chemotherapeutic, Paclitaxel [94]. CD33 is a transmembrane protein involved in cellular adhesion and Aβ clearance processes; reduced expression of CD33 has been shown to result in increased uptake of Aβ42 [109, 114, 163]. Nonetheless, inhibition of the resistant DU145-TXR cells resulted in a 33% greater change in Aβ fluorescence relative to the change in DU145 cells, again strongly suggesting that inhibition of P-gp transport resulted in low levels of Aβ efflux through P-gp with a concomitant increase in Aβ accumulation in these cells.
3.4. Conclusion

Through the combination of computational simulations, kinetic ATP hydrolysis measurements of purified P-glycoprotein, and transport assays in a human cellular environment, we have shown here that P-gp is able to transport two different monomeric forms of Aβ. While there is a growing body of evidence that P-gp plays an important role in the clearance of Aβ across the BBB, such conclusions are beyond the scope of this study, other than to show that we can observe transport of Aβ peptides through P-gp in TMD simulations [125, 127, 164]. Adding Aβ to the list of known substrates indicates that P-gp can transport much larger molecules than was previously thought. Given the clinical importance of P-gp and of other ABC transporters, we believe that the ability of human efflux pumps to transport large ligands, and the mechanism by which they do so, warrants further study.

3.5. Methods

3.5.1. Docking Aβ to Human P-gp

Aβ structures were docked to the drug binding domains (DBDs) of P-glycoprotein in the open-to-the-cytoplasm conformation (derived from the homologous 4KSB structure) of human P-gp using AutoDock Vina as described previously (Figure 3.2.3) [39, 59, 64, 92]. Ligand interactions were limited to the cytoplasmic extensions of the transmembrane helices and the transmembrane sections of P-gp and used an exhaustiveness of 128 (the default exhaustiveness or number of replica docks for Vina is set at 8). Ligand binding to nucleotide binding domains (NBDs) was not investigated. The resultant ligand docking positions were ranked by predicted binding affinities; the conformational pose with the highest predicted affinity was used as a starting point for molecular dynamics (MD) simulations, except when indicated in the text.
Three different structures of Aβ were docked to the DBDs of P-gp: 2LFM, a partially folded solid state NMR structure of Aβ40 in an aqueous environment, docked with a predicted affinity of -7.2 kcal/mol (Figure 3.2.2A) [140]; 2M4J, a 40 residue Aβ fibril derived from AD brain tissue docked with a predicted affinity of -7.1 kcal/mol (Figure 3.2.2B) [138]; and 1IYT, a solid state NMR structure of Aβ42 in an apolar microenvironment, with a predicted affinity of -7.2 kcal/mol (Figure 3.2.2C) [138]. As a control peptide, every residue in the highest affinity docking pose of Aβ40 fibril 2LFM was mutated into an arginine, creating the Polyarginine 42 peptide (Figure 3.2.2D). Docking studies were performed by James McCormick.

3.5.2. Transport of Aβ Through P-gp in Molecular Dynamics Simulations

To facilitate the efflux of substrates across the cell membrane, ATP-driven ABC-transporter proteins undergo large conformational changes powered by the binding and hydrolysis of ATP. These conformational changes switch the drug binding domains (DBDs) of the transporter from “open to the cytoplasm” (inward facing) to “open to the extracellular space” (outward facing) [93, 165]. Such cycling has been hypothesized for P-gp and has previously been shown by us in computational simulations to transport small-molecule, drug-like ligands from the cytoplasmic membrane leaflet to the extracellular leaflet and extracellular space [25]. The modeled, putative catalytic cycle of P-gp therefore reflects an hypothesized sequence of conformational changes for ABC transporters and has allowed us to visualize substrate transport driven by P-glycoprotein. These previous studies have allowed us to investigate P-glycoprotein-driven movement of small drug-like molecules across the membrane. These computational simulations have been extended here to the larger, polypeptide substrates, Aβ40 and Aβ42.
To model a putative catalytic transport cycle of P-gp, we used crystal structures of P-gp homologues in various conformations as in Wise 2012 and McCormick et al. 2015 [25]. Because these structures were determined from crystals, the conformations represented by these structures are relatively stable, representing relatively low energy conformations of the protein. During TMD simulations, small forces were applied to selected Ca atoms of P-gp to direct the movement of protein domains toward the respective target coordinates. The putative catalytic transport cycle of P-gp we used follows the sequence of conformational states based on earlier work [25, 64]: (1) a conformation with the DBDs wide open to the cytoplasm (derived from 4KSB); (2) a conformation with the DBDs slightly open to the cytoplasm (derived from 3B5X); (3) a conformation with fully engaged NBDs and DBD opened to the exterior (derived from 2HYD); and (4) a final conformation with NBDs in an ATP hydrolysis transition state and the DBDs fully open to the extracellular space (derived from 3B5Z) (Figure 3.2.3). Using these TMD simulations, we guided P-gp through a putative transport cycle and included Aβ40 (2LFM, 2M4J) or Aβ42 (1IYT) in the DBDs on the cytoplasmic side of the membrane [64, 138-140].

The inward facing structure of the mouse P-gp (4KSB) has fully disengaged NBDs with its transmembrane DBDs oriented in an inward facing state [92]. The structure of MsbA from *Vibrio cholerae* (3B5X) has disengaged NBDs, and DBDs partially open to the cytoplasm. The structure of SAV1866 from *S. aureus* (2HYD) has engaged NBDs and its DBD open to the outside [93, 166]. The structure of MsbA from *S. typhimurium* (3B5Z) also has fully engaged NBDs with its DBD open to the outside but may represent a post-hydrolysis transition state since crystallization conditions included an MsbA - ADP-vanadate complex. Using the structures in the aforementioned sequence, we previously simulated the conformational changes of a putative catalytic transport cycle using models of human P-gp and TMD simulations [25, 64] based on
structures from [76, 167]. TMD Simulations and analyses were performed by James McCormick and by Lauren Ammerman.

3.5.3. Preparation of the Aβ42 Synthetic Peptide

3.5.3.1. Monomerization of Aβ42 for ATPase assays

The Aβ42 synthetic peptide was purchased from GenicBio Limited, PRC (sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA). The peptide had a molecular weight of 4514.14 g/mol and was judged to be 95.40% pure by HPLC. To monomerize the protein, lyophilized Aβ42 was removed from storage at -80°C and allowed to equilibrate at room temperature for 30 minutes to avoid condensation upon opening the vial. In a fume hood, 1 mg of the lyophilized Aβ42 peptide was resuspended in 300 µl of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich). The mixture was sonicated and vortexed thoroughly to ensure proper solvation. The solution was then centrifuged at 10,000 rpm for 5 min and the supernatant was moved to a clean tube. Centrifugation was repeated once more to remove any insoluble materials. The solution was then aliquoted into separate vials where the HFIP was allowed to evaporate in the fume hood overnight. The desiccated pellets were stored at -20°C. For use in ATPase assays, the samples were resuspended in a 1:4 mixture of dimethyl sulfoxide (DMSO) and sterile water. This procedure was performed by James McCormick. We thank Professor Heng Du (UT Dallas Biological Sciences) for providing the procedures for monomerizing the Aβ peptides.

3.5.3.2. Preparation of fluorescently labeled Aβ42 for cell culture assays

Fluorescent (HiLyte™ Fluor 488) labeled Aβ42 was purchased for cell culture assays from Anaspec (HiLyte™ Fluor 488 –
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVMGVVIA). This peptide had a molecular weight of 4870.5 g/mol, absorption/emission wavelengths of 503/528 nm, and was judged to be >= 95% pure by HPLC (CAT# AS-60479, LOT# 1958003). To prepare the Aβ peptide for cell culture assays, 0.1mg of the peptide was dissolved in 30 uL of 1% (w/v) ammonium hydroxide in sterile water and filtered using a 0.45 um pore filter [168]. Once thoroughly dissolved and mixed, 380 uL of Phosphate Buffered Saline (PBS) solution were added to the peptide-ammonium hydroxide solution (final NH₄OH 0.073%). After mixing thoroughly again, the peptide solution was aliquoted into 50 µL aliquots and frozen until use. When using the peptide solution for cell culture experiments, the peptide containing aliquot was thawed and vortexed immediately before use. This procedure was performed by Lauren Ammerman and John G. Wise.

3.5.4. Accumulation of Fluorescently Labeled Aβ42 in DU145 and DU145-TXR Cells

Cell Culture work, Confocal Microscopy, and analyses were performed by Lauren Ammerman. Multidrug-resistant (MDR) DU145-TXR prostate cancer cells have been previously shown to overexpress P-glycoprotein [94]. These MDR DU145-TXR cells (kindly provided by Evan Keller, Univ. of Michigan) were derived from drug sensitive DU145 cancer cells by culturing in the presence of the chemotherapeutic paclitaxel to create the P-gp overexpressing cell line DU145-TXR [94]. Both DU145 and DU145-TXR cells were grown in complete media consisting of RPMI-1640 with L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37 °C using 5% CO₂. The drug-resistant line DU145-TXR was maintained under positive selection pressure by supplementing complete media with 10 nmol/L paclitaxel. Both cell lines were grown and seeded on collagen-treated flasks and plates (Collagen Type I, Corning).
To assess the accumulation of fluorescently labeled Aβ42 in both cell lines, cells were trypsinized from monolayers and seeded at 60,000 cells per well in 24 well plates in complete RPMI media. Prior to seeding, a sterilized glass coverslip was placed in each well, and each well was treated with a working solution of 0.01 mg/mL collagen Type I (Corning) in 0.02 N Acetic Acid for 10 minutes and rinsed with PBS. After 24 hours of incubation at 37 °C, the media was removed and replaced with fresh complete RPMI media. The cells were dosed with 1 µM of the P-gp inhibitor tariquidar (TQR), 1 uM of HiLyte-488-Aβ42, a combination of both, or 2% DMSO media as a control, and incubated at 37 °C and 5% CO₂ for 16 hours [169]. The final concentration of ammonium hydroxide in each well was kept at approximately 0.0015 % and was matched in controls to ensure identical treatment of all cell samples. If there was any leftover Aβ in the thawed aliquot, the excess Aβ was not re-frozen, but was discarded to avoid aggregation that can be induced by freeze-thawing. After 16 hours of incubation, media was removed, and cells were gently washed with ice-cold PBS. Cells were then fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature (approximately 27°C). Cells were then stained with DAPI in PBS for 10 minutes, and subsequently washed twice with cold PBS. Each coverslip was removed from the well and mounted on a glass slide using Fluoromount G mounting fluid.

After drying slides overnight, fluorescence-confocal microscopy was performed on a Zeiss LSM800 microscope using Plan-Apochromat 20x and 40x/1.3 oil-immersion objectives. Fluorescence of HiLyte488-Aβ42 or DAPI was measured using the pre-programmed EGFP and DAPI filters and the Zeiss ZEN 3.3 software. Specifically, the EGFP filter uses excitation/emission wavelengths of 488/509 nm, and DAPI uses excitation/emission of 353/465 nm. Detection wavelengths range from 495-565 nm for EGFP, and 410-470 nm for DAPI.
Images taken at 40X were used to quantify HiLyte488-Aβ42 fluorescence; the inclusion of the Bright Field channel allowed us to identify – and subsequently exclude from quantification – any extracellular HiLyte488-Aβ42 fluorescence.

Experiments were performed in duplicate, with two independent trials, and two samples per treatment in each trial. At 20X magnification, 12 images per individual slide were taken. At 40X magnification, 6 images per individual slide with HiLyte488-Aβ42 treatment were taken and used for quantification; at least three images were taken for the TQR-only or DMSO control slides to confirm the lack of observable HiLyte488-Aβ42 fluorescence, which was confirmed initially by the images taken at 20X magnification. Thus, for quantification of HiLyte488-Aβ42 fluorescence, data are presented as the mean and standard deviation, with 24 replicates per treatment, 12 replicates from each individual trial. All images were taken before analysis was performed; all images captured at 40X magnification of the slides treated with HiLyte488-Aβ42 were included in the analysis. Z-stacks of the HiLyte488-Aβ42-treated cells, with or without TQR, were taken at 40X magnification to visualize any intracellular accumulation of HiLyte488-Aβ42. A set of Z-stacks were taken with the Bright Field channel to aid visualization of the DU145 or DU145-TXR cell boundaries.

Quantification was performed using FIJI ((Fiji Is Just) ImageJ), NIH, Bethesda, Maryland, USA) [149, 151, 152] on the raw, unedited CZI files obtained from the microscope. To quantify fluorescence with ImageJ, raw CZI image files were imported using the Bio-Formats plugin [150]. Analysis of HiLyte488-Aβ42 fluorescence with ImageJ was automated using ImageJ macros (included in Supplementary Files). Using a copy of the raw green channel image, regions of HiLyte488-Aβ42 fluorescence were “thresholded” as follows: (1) used the default “Subtract Background” function (rolling ball radius of 10 pixels (px)); (2) applied the default
Unsharp Mask (radius 1 px, mask = 0.60 sigma) to define the edges of fluorescent areas; (3) thresholded the image (minimum 15, maximum 255) to determine which areas will be measured; (4) converted the thresholded image to a binary mask; (5) saved the binary mask as a TIF image. Through this process, a binary mask is created in which areas of thresholded HiLyte488-Aβ42 fluorescence are black, and all other areas are white. The binary mask was then used to define regions for measurement on the original, raw green channel image. Using the binary mask to define regions for measurement, areas of thresholded HiLyte488-Aβ42 fluorescence in the raw green channel image were measured using the default “Analyze Particles” function of ImageJ. Using the previously created binary mask of each image, the mean background intensity of each raw green channel image was measured by selecting the inverse of the HiLyte488-Aβ42 areas, and then using the default “Measure” function of the software. Aβ Fluorescence was quantified as a measure of the “corrected mean fluorescence intensity” using the following formulae and is reported in arbitrary units of fluorescence.

\[
\text{(3.1)} \quad \text{Integrated Density}_{\text{area}} = \text{Sum(value of each pixel in area)} \times 0.024329 \, \mu\text{m}^2\text{pixel}^{-1}
\]

\[
\text{(3.2)} \quad \text{Mean Intensity} = \frac{\text{Integrated Density}_{\text{area}}}{N_{\text{pixels in area}}}
\]

\[
\text{(3.3)} \quad \text{Corrected Mean Intensity}_{\text{Aβ}} = \text{Mean Intensity}_{\text{Aβ}} - \text{Mean Intensity}_{\text{background}}
\]

Once the initial analysis was completed using ImageJ, images were examined for potential extracellular HiLyte488-Aβ42 fluorescence using the Bright Field channel overlaid with the green channel as a guide. In the event of extracellular particles, we (1) created a copy of the original binary mask for that image; (2) manually removed the extracellular particle from the
binary mask copy; (3) ran the measuring and analysis functions using the ‘corrected’ binary
mask to redirect measurements. Any extracellular areas were manually removed from the binary
mask, and the image was re-analyzed using the same macro. This procedure was performed for
10/48 total images; 6/11 were from HiLyte488-Aβ42 images; 4/11 were from HiLyte488-Aβ42 +
TQR images. This resulted in an average change of 0.01 ± 0.42 a.u. from the original values. To
compare the levels of HiLyte488-Aβ42 fluorescence between treatments, we report the Corrected
Mean Intensity in arbitrary units. Data was analyzed using a two-tailed T test with equal variance
and GraphPad Prism version 7 for Windows, GraphPad Software, San Diego, California USA,

The DAPI-GFP-BF merge images were enhanced for inclusion in figures, and viewing in
a small format, using the Zen Blue 3.3 as follows – Bright field channel, levels adjusted using the
‘Min Max’ default setting to enhance contrast and cell outlines; DAPI, levels adjusted using the
‘Best Fit’ default setting to enhance contrast; GFP (Green channel for HiLyte488-Aβ42), levels
adjusted by setting the maximum to 149. Zen Blue 3.3 was used to export high resolution files of
each image for assembly into figures. Microscopy figures were assembled using Adobe
Illustrator. The full protocol, with the accompanying Image J macros, can be accessed at
Protocols.io (dx.doi.org/10.17504/protocols.io.brvem63e).

3.5.5. ATPase Activity Assays with P-gp in Nanodiscs and Micelles

ATPase activity assays and associated work was performed by Gang Chen. Analyses were
performed by James McCormick.
3.5.5.1. Purification of Murine P-gp

Murine cys-less P-gp was used for all ATPase activity assays [3, 144, 170, 171]. Protein purification of P-gp and ATP hydrolysis assays were performed as described in Delannoy et al 2005 and Brewer et al. 2014 with some modifications as described below [3, 170].

3.5.5.2. Preparation of P-gp in Micelles

P-gp was expressed in *Pichia Pastoris* GS-115. To isolate P-gp from its native membrane and embed it in detergent micelles, 80 mL of frozen cell pellets were thawed in a 37 °C water bath, and protease inhibitors (160 µL pepstatin A, 32 µL Leucine, 16 µL chymostatin, 800 µL of 200 mM PMSF and 800 µL of 200 mM DTT) were subsequently added. Cells were then broken open using 175 mL of glass beads and a BeadBeater (Biospec products). The BeadBeater was filled to the top with buffer containing 30% glycerol, 50 mM Tris, 125 mM NaCl, 10 mM imidazole (pH 8.0). To prevent the samples from overheating during bead beating, ice with rock salt was used. Samples were spun at 10,000 rpm for 30 min at 4 °C to remove debris, nuclei, mitochondria and unbroken cells using a Beckman Avanti JXN-26 centrifuge. The supernatants were then subjected to a fast spin at 45,000 rpm for 45 min at 4 °C using Beckman Optima XPN-80. The resultant pellets (which contain P-gp) were washed with microsome wash buffer (20% glycerol, 50 mM Tris, pH 7.4), and resuspended in Tris buffer (30% glycerol, 50 mM Tris, 125 mM NaCl, 10 mM imidazole, pH 8.0). Nickel-NTA columns were used to capture P-gp engineered with His-tag.

Microsomes containing P-gp were diluted with Tris buffer (20% glycerol, 50 mM Tris, 50 mM NaCl, 10 mM imidazole, pH 8.0) to 2 mg/mL, and 0.6% n-Dodecyl β-D-maltoside (DDM, w/v) (Sigma-Aldrich) and 0.01% lysophosphatidylcholine (lyso-PC, w/v) were added. Then, the sample solution was sonicated in an ice-cold water bath (model) for 5 cycles of 5 min. Samples
were then spun down at 20,000 rpm for 30 min at 4°C using a Beckman Optima XPN-80 centrifuge to remove undissolved microsomal proteins. The supernatants were then applied to a Ni-NTA gravity column (QIAGEN), and incubated for 30 min at 4°C. Flow-through was collected at 2 mL/min, and the column was washed with 20 bed volume of wash buffer (20% glycerol, 50 mM Tris, 50 mM NaCl, 20 mM imidazole, pH 7.5) at 1mL/min, followed by 10 bed volume of the second wash buffer (20% glycerol, 50 mM Tris, 50 mM NaCl, 40 mM imidazole, pH 7.5) at 1 mL/min. Protein was eluted with buffer (20% glycerol, 50 mM Tris, 50 mM NaCl, 300 mM imidazole, pH 7.5) for 3 bed volumes at 0.5 mL/min. Both buffers for the wash and elution were supplemented with 0.6% DDM (w/v) and 0.01% lyso-PC (w/v) if not otherwise specified. The eluates were concentrated to about 150 µL using Amicon 100K centrifugation filters (MilliporeSigma) at 4°C, and stored at -80°C.

3.5.5.3. Reconstitution of P-gp into Nanodiscs

P-gp was incubated with a 10x molar excess of membrane scaffold protein (MSP) and a 500x molar excess of 40% L-alpha-phosphatidylcholine (PC) from soybean (Sigma) for 1 hour at room temperature with gentle agitation to facilitate formation of nanodiscs [172]. Biobeads SM-2 (BioRad) were presoaked in methanol, washed with a large amount of water, equilibrated with equilibration buffer (20% glycerol, 50 mM Tris-HCl pH 7.5, 50 mM NaCl), and finally added at a ratio of 1.4 g/mL to the assembly mix to remove detergent. After addition of Biobeads SM-2, the mixture was incubated for 1.5 hours at room temperature with shaking to remove detergent from the crude nanodisc sample. The Biobeads were removed from the crude nanodiscs by piercing the bottom of the centrifuge tube with a 25 gauge needle and centrifuging at 1000xg for 1 minute. Empty discs were removed by Ni-NTA column chromatography, utilizing the histidine–tag at P-gp. Six bed volumes of column wash buffer (20% glycerol (v/v) 50 mM Tris-
HCl pH 7.5 at RT, 50 mM NaCl, 20 mM imidazole) were then applied. Purified nanodiscs were eluted from the column by applying 1 bed volume elution buffer (20% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 4°C, 50 mM NaCl, 300 mM imidazole). Samples were analyzed by gradient SDS-PAGE and coupled enzyme assays.

3.5.5.4. ATPase Activity Assays

Briefly, ATP hydrolysis by P-gp was coupled to the oxidation of NADH to NAD$^+$ by two enzymes, pyruvate kinase and lactate dehydrogenase, as described in [3]. The coupled enzyme assay cocktail included 50 mM Tris, pH 7.5, 24 mM MgSO$_4$, 20mM KCl, 1.94 mM phosphoenolpyruvate (PEP), 0.058 mg/mL pyruvate kinase, 0.0288 mg/mL lactate dehydrogenase, 1.13 mM NADH, and 4 mM ATP. The absorbance decrease of NADH at 340 nm was recorded using a BioTek Eon plate reader BioTek. The ATPase activity of P-gp was directly correlated with the rate of NADH oxidation. An extinction coefficient of 6220 M$^{-1}$cm$^{-1}$ at 340 nm was used for calculations of NADH oxidation with a measured path length of 0.6 cm.

Aβ$_{42}$ was incubated with P-gp for 30 min at 37°C before the addition of coupled enzyme assay cocktail.

In the activity assays reported here, 15 µg of purified P-gp was used for nanodiscs, and 20 ug of purified P-gp was used for micelles. A molar ratio of 1:18, P-gp:Aβ$_{42}$, was used for experiments with both nanodiscs and micelles - for P-gp in mixed micelles, 712 nM P-gp to 12.8 µM Aβ$_{42}$ was used, and for P-gp in nanodiscs, 534 nM of P-gp to 9.6 µM of Aβ$_{42}$ was used. The basal ATPase activity of P-gp in mixed micelles was 51 ± 3 nmol/min/mg, and the Verapamil (VPL)-stimulated activity was 106 ± 7 nmol/min/mg. The basal ATPase activity of P-gp in nanodiscs was 131 ± 9 nmol/min/mg, and the VPL-stimulated activity was 390 ± 14 nmol/min/mg. A VPL concentration of 150 µM was used, as in [173]. ATPase activity
experiments to measure basal and stimulated activity were performed with n = 4. For ATPase
activity experiments with VPL and/or Aβ42, experiments were performed with n = 3. One
protein preparation was used for each of the different conditions (micelles and nanodiscs).
Resultant data represent the mean ± one standard error of the mean.
CONCLUSION

In this work, we have presented three complete projects focusing upon two dynamic mechanisms of multidrug resistance – the ABC transporter P-gp, and the RND transporter MtrD. In Chapter 1, we demonstrated that substrate transport through the periplasmic cleft of MtrD depends upon a combination of diffusion, gated access to areas with variable charge and lipophilicity isosurfaces, and conformational changes of the surrounding structure. Our results suggest that multiple transport pathways may exist within the periplasmic cleft of MtrD. In Chapter 2, using a suite of comprehensive in vitro and in silico methods, we reported a 13% global hit rate for the identification of P-gp inhibitors with a < 15 μM cutoff – compounds 70, 78, 96, 97, 101, 103, 111, 122, and 124. From these 9 candidates, we have identified 6 novel P-gp inhibitors for lead optimization– compounds 70, 78, 96, 97, 101, and 111. These compounds were not transport substrates of P-gp itself. This work presents an enhancement of traditional virtual-assisted screening methods by a significant factor. Lastly, in Chapter 3, we used MD simulations, cell-based assays, and kinetic measurements to show that P-gp can transport the Alzheimer’s associated Aβ peptides. Our data indicate that P-gp can transport much larger molecules than was previously thought. Given the clinical importance of P-gp and of other ABC transporters, we believe that the ability of human efflux pumps to transport large ligands, and the mechanism by which they do so, warrants further study. Finally, the user “lammerman” accounted for a whopping 11% of the total usage of Maneframe 2 computational resources from
its birth in 2017, to lammerman’s graduation in 2021. Lammerman accounts for 1.848 million jobs of 16.77 million jobs submitted to Maneframe 2 as of this writing.
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