

DECIPHERING THE MOLECULAR MECHANISMS UNDERLYING THE TRANSPORT OF ANTI-TUMOR ALKALOIDS BY P-GLYCOPROTEIN

by

GERSHON A.K. MENSAH

(Under the Direction of Arthur G. Roberts and Michael G. Bartlett)

ABSTRACT

P-glycoprotein (Pgp), an evolutionary conserved ABC transporter predominantly localized in cell membranes, acts as a gatekeeper regulating the passage of various molecules, including drugs and toxins. The protective physiological function of Pgp against toxic substances can induce multi-drug resistance in chemotherapy. As a result, Pgp-mediated transport presents a formidable hurdle to drug development. The membrane environment plays a significant role in influencing the activity of Pgp. Cholesterol derivatives in the membrane can be flip-flopped between membrane leaflets by Pgp, accounting partially for the traditionally high basal ATPase activity reported. Second, cholesterol derivatives indirectly modulate Pgp activity by providing a conducive conformation of the protein necessary for transport and aiding substrate partitioning into the inter-leaflet space, where Pgp's binding cavity resides. While inhibiting Pgp is a logical strategy to combat drug resistance, the lack of approved Pgp inhibitors for cancer treatment and the limited success of repurposed FDA-approved drugs as inhibitors in clinical studies present obstacles. An alternative involves redesigning existing drugs to be non-Pgp-substrates, leading to more cost-effective drug design. Interestingly, Pgp-mediated transport rates can differ between drugs of the same class. To better understand this phenomenon and set the tone for rational drug design, we investigated some of the antitumor alkaloids such as the camptothecin analogs (CPTs):

topotecan (TPT), SN-38, and irinotecan (IRT); and vinca alkaloids (VAs): vinblastine (VBL) and vinorelbine (VRL) and vincristine (VCR). The action of Pgp has limited the efficacy of these otherwise highly potent chemotherapeutic agents. Conformationally-gated models underlying Pgp-mediated transport of these classes of drugs are presented. Additionally, specific functional groups on the CPTs and VAs that likely interact with Pgp were identified. Our study, when coupled with existing structure-activity relationship investigations, offers a promising path for creating enhanced drug derivatives that could withstand Pgp-mediated efflux.

INDEX WORDS: P-glycoprotein, ABC transporter, NMR spectroscopy, fluorescence spectroscopy, AFM imaging, Cholesterol, Conformational changes, Cancer, Multidrug resistance,

**DECIPHERING THE MOLECULAR MECHANISMS UNDERLYING THE
TRANSPORT OF ANTI-TUMOR ALKALOIDS BY P-GLYCOPROTEIN**

By

GERSHON A.K. MENSAH

B.Sc. (Biochemistry), 2016

Kwame Nkrumah University of Science and Technology, Ghana.

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2023

© 2023

Gershon A. K. Mensah

All Rights Reserved

**DECIPHERING THE MOLECULAR MECHANISMS UNDERLYING THE
TRANSPORT OF ANTI-TUMOR ALKALOIDS BY P-GLYCOPROTEIN**

by

GERSHON A.K. MENSAH

Major Professors: Arthur G. Roberts (Ph.D.)
Michael G. Bartlett (Ph.D.)

Committee Members: Xiaoqin Ye (Ph.D.)
Jeffrey L. Urbauer (Ph.D.)
Robert M. Gogal (Ph.D.)

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
December 2023

DEDICATION

This dissertation is dedicated to my best friend and wife, Eunice Hilda Ampomah Mensah, my little sunshine, Ranielle O. A. Mensah, and my mom, Sophia Aba Rockson. Your constant prayers, encouragement and support has been the driving force behind my academic pursuits. This achievement is as much yours as it is mine.

ACKNOWLEDGEMENTS

“Strive for your best always, or risk living with regret” – Gershon Mensah

To the love of my life, Eunice Hilda Ampomah Mensah, whose encouragement and understanding have been my pillars of strength throughout this academic journey. Your belief in me fueled my determination, and your sacrifices allowed me the time and space to pursue my passion. You brought out the tenacity in me that I never knew existed. You are simply the best!

To my dearest mom, Sophia Aba Rockson, whose boundless love and wisdom have shaped me into the person I am today. Mama, your constant encouragement, endless prayers, and unyielding belief in my abilities have been the driving force behind my academic pursuits. I could not have done this without you.

To my precious little princess, Ranielle O. A. Mensah, who has been my source of inspiration and joy. Your innocence, smiles, and funny little giggles have provided a welcome respite from the challenges of academia, reminding me that there is a much bigger picture to life than I can ever imagine. I cannot wait to hold you in my hand!

To my lab mates and friends, too numerous to list here, thank you for your suggestions, and most importantly, your support. I could not have done this without you all.

To my advisors, Dr. Roberts and Dr. Bartlett. My sincere heartfelt gratitude for your unwavering support, guidance, understanding, and patience over the last five years. I appreciate the numerous opportunities you provided throughout my graduate career. God richly bless you!

To my committee members. Thank you for your advice, your encouragement, valued input, and understanding. The impact you have made on my graduate career has been priceless and would forever remain part of me.

TABLE OF CONTENTS

LIST OF TABLES	VIII
LIST OF FIGURES	IX
CHAPTER 1	1
1.1 ABSTRACT	2
1.2 EXPRESSION, LOCALIZATION, AND FUNCTION OF P-GLYCOPROTEIN	3
1.3 THE ARCHITECTURE OF P-GLYCOPROTEIN.....	17
1.4 P-GLYCOPROTEIN-MEDIATED SUBSTRATE TRANSLOCATION MODELS.....	23
1.5 OVERCOMING P-GLYCOPROTEIN-MEDIATED MULTI-DRUG RESISTANCE.....	30
1.6 CONCLUSION	36
1.7 FIGURES.....	37
CHAPTER 2	44
2.1 ABSTRACT	45
2.2 INTRODUCTION	46
2.3 EXPERIMENTAL PROCEDURE	49
2.4 RESULTS.....	58
2.5 DISCUSSION.....	67
2.6 FIGURES.....	74
2.7 SUPPLEMENTARY INFORMATION.....	85
CHAPTER 3	87
3.1 ABSTRACT	88

3.2 INTRODUCTION	89
3.3 EXPERIMENTAL REAGENTS AND METHODS	92
3.4 RESULTS.....	102
3.5 DISCUSSION.....	111
3.6 FIGURES.....	119
3.7 SUPPLEMENTARY INFORMATION.....	129
CHAPTER 4	130
4.1 ABSTRACT	131
4.2 INTRODUCTION	132
4.3 EXPERIMENTAL PROCEDURE	135
4.4 RESULTS.....	140
4.5 DISCUSSION.....	145
4.6 FIGURES.....	152
CHAPTER 5	160
5.1 SUMMARY	160
5.2 FUTURE DIRECTIONS.....	173
6.0 REFERENCE.....	176

LIST OF TABLES

	Page
Table 2.1: Kinetic parameters from Pgp-coupled CPTs-induced ATPase activity.....	84
Table 3.2: Kinetic parameters deduced by fitting the rate of Pgp-coupled ATPase activity curves over a range of vinca alkaloid concentrations.....	128
Table 3.2: Kinetic parameters determined by fitting Pgp-VAs binding affinity curves.....	94

LIST OF FIGURES

	Page
Figure 1 1 A dendrogram of cluster of existing human ATP-binding cassette (ABC) transporter genes	37
Figure 1 2 Effect of xenobiotics on different transcription factors that influence the expression of P-glycoprotein.....	38
Figure 1 3 Illustration of the potential intracellular localization, trafficking, and recycling of P-glycoprotein	39
Figure 1 4 The distribution of P-glycoprotein in various tissues.....	40
Figure 1 5 The open-inward and open-outward conformations of P-glycoprotein in Protein Data Bank	41
Figure 1 6 The substrate binding and conformational transport model of P-glycoprotein.....	42
Figure 1 7 The crosstalk region between the TMD and the NBD of P-glycoprotein	43
Figure 2 1 The basic structure of the camptothecins analogs	74
Figure 2 2 The effect of TPT, SN-38, and IRT on Pgp-mediated ATP hydrolysis	75
Figure 2 3 The binding affinity of TPT, SN-38, and IRT to Pgp determined by the quenching of intrinsic protein fluorescence	76
Figure 2 4 Camptothecin-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence	78
Figure 2 5 The interactions of camptothecin analogs with Pgp were investigated using STDD NMR spectroscopy.....	80

Figure 2 6 The effect of TPT, SN-38, and IRT on the height distributions of Pgp for the C-domain protrusions determined via AFM	81
Figure 2 7 A cartoon depiction of a conformationally-gated model of Pgp conformations in the lipid bilayer in the presence of TPT, SN-38, and IRT	83
Figure 3 1 The basic structure of the Vinca Alkaloids (VAs)	119
Figure 3 2 The effect of the vinca alkaloids on Pgp-mediated ATP hydrolysis	120
Figure 3 3 The binding affinity of vinca alkaloids to Pgp determined by the quenching intrinsic tryptophan fluorescence by ligands	121
Figure 3 4 Vinca alkaloid-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence	123
Figure 3 5 The functional groups of vinca alkaloids involved in its interactions with Pgp investigated by STDD NMR.....	125
Figure 3 6 The effect of vinca alkaloids on the height distributions of Pgp at the cytosolic domain (C-domain) analyzed deduced AFM imaging.....	126
Figure 3 7 Model illustrating VA-induced activation of Pgp-mediated ATP hydrolysis and transport	127
Figure S3 1 Analyses of the height distribution of Pgp in the lipid bilayer.....	129
Figure 4 1 The structures cholesterol and some of the common oxidized derivatives	152
Figure 4 2 The effect of cholesterol and its derivatives on Pgp-mediated ATP hydrolysis	153
Figure 4 3 The effect of the derivatives of cholesterol on verapamil-stimulated Pgp-mediated ATP hydrolysis	154
Figure 4 4 The effect of cholesterol (CHL) composition in liposomes on the conformation of Pgp probed by acrylamide quenching of protein fluorescence	155

Figure 4 5 The impact of 7-ketocholesterol (7KC) composition within liposomes on Pgp conformation	156
---	-----

CHAPTER 1

LITERATURE REVIEW:

AN INSIGHTS INTO STRUCTURE, PHYSIOLOGICAL FUNCTION, REGULATION, AND THE ROLE OF P-GLYCOPROTEIN IN MULTIDRUG RESISTANCE^{1 1}

¹ Mensah, GAK., and Roberts, AG (2023). To be submitted to *American Chemistry Society*.

1.1 ABSTRACT

P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) superfamily, which utilizes ATP to transport a wide range of structurally diverse compounds out of cells. The primary function of Pgp is to protect tissues from harmful substances by limiting the cellular uptake of xenobiotics. Consequently, Pgp is expressed ubiquitously but is found at higher levels in the gastrointestinal tract, liver, kidneys, and several blood-tissue barriers. Due to their protective role, disease phenotypes can arise due to alterations in the expression levels of P-glycoprotein, which are influenced by various enzymes, transcription factors, and inflammatory mediators. Pgp, encoded by the ABCB1 gene, is a 170 kDa monomer protein comprising twelve transmembrane helices and two nucleotide-binding domains. This transporter undergoes significant conformational changes through ATP binding and catalysis to enable transport. While Pgp is crucial in maintaining homeostasis, it can also restrict many therapeutic compounds from reaching their targets and has become a significant obstacle in pharmaceutical development. Studies on the structure-activity relationship have identified molecular features that contribute to substrate binding; however, the broad specificity of Pgp has made it challenging to design therapeutics that target or circumvent transport. This review details the expression, localization, physiological structure and function, regulation, and the role Pgp plays in the development of multi-drug resistance phenotypes in chemotherapy.

1.2 EXPRESSION, LOCALIZATION, AND FUNCTION OF P-GLYCOPROTEIN

1.2.1 The Expression of P-glycoprotein

The ATP-binding cassette (ABC) superfamily of transporters consists of a family of 49 energy-dependent integral membrane proteins that are mostly conserved throughout evolution (Robinson & Tiriveedhi, 2020). ABC transporters use ATP hydrolysis to power the uptake and efflux of solutes across cellular membranes in prokaryotes (and efflux only in eukaryotes). The ABC superfamily is noteworthy due to its ubiquity (being present in multiple species) and its abundance in normal and pathological cells or organs. For example, ABC transporters account for about 5% of the entire genome of *E. coli* (Linton & Higgins, 1998). In plants, the extensive range of ABC transporters serves a dual purpose: they facilitate the transportation of xenobiotics, hormones, secondary metabolites, metals, and lipids and are also involved in the evolution of seed size (Orsi & Tanksley, 2009).

The human genome encodes 49 genes for ABC proteins designated to one of the seven subfamilies labeled A to G, as shown in Figure 1.1 (Vasiliou et al., 2009). Each subfamily plays one or more pivotal roles in cellular processes. The ABCB1 (MDR1) gene located on chromosome 7q21.12 encodes for P-glycoprotein (Pgp), which belongs to subfamily B of the ABC family in humans (Callen et al., 1987; Feng & Xie, 2016). The ABCB1 gene is relatively large, consisting of about 32 exons (ABCB1 ATP Binding Cassette Subfamily B Member 1 [Homo Sapiens (Human)] - Gene - NCBI, n.d., p. 1), ranging from 49 to 587 base pairs (bp). The full-length cDNA of MDR1 can span between 3.4 to 4.5 kb (Ambudkar et al., 2003; Ueda et al., 1987). While more than 50 single nucleotide polymorphisms (SNPs) have been identified on ABCB1, only a few, including A61G, T307C, G1199A, G2677T, and G2955A, lead to amino acid substitutions (Ambudkar et al., 2003). Importantly, the SNPs resulting in amino acid substitutions have demonstrated minimal influence on drug pharmacokinetics (Ambudkar et al., 2003). The relatively

strong resistance of Pgp to site-directed mutagenesis in both the transmembrane (TMD) and nucleotide-binding domains (NBD) underscores the minimal impact of SNPs on the protein's structure and overall functionality (Loo & Clarke, 1995; Swartz et al., 2013). To date, there have been no reports of point mutations resulting in a complete loss of function of ABCB1 (Zawadzka et al., 2020). An exception among SNPs that have been demonstrated to impact drug disposition considerably (Ambudkar et al., 2003; Feng & Xie, 2016) and raise the risk of various diseases is the MDR1(C3435T) located on exon 26 (Y.-H. Li et al., 2006). This polymorphism has been observed to be a "silent" mutation that does not change the amino acid sequence. It is present in approximately 26% of the Caucasian population, around 17% of the Asian population, and only about 2.3% of the African population (Ambudkar et al., 2003; Feng & Xie, 2016). MDR1(C3435T) polymorph has been observed in epileptic patients with poor response to antiepileptic drugs (Kwan et al., 2007). On the contrary, other studies find no correlation between the MDR1(C3435T) polymorph and drug disposition (Ambudkar et al., 2003; Bournissen et al., 2009). Thus, the apparent multi-drug resistance observed in MDR1(C3435T) may have resulted from polymorphism of a neighboring gene that appears to alter or influence the expression of Pgp (Ambudkar et al., 2003). On the contrary, there have been reports of point mutations that reduce Pgp activity or alter drug transport function (Hsin et al., 2020). A point mutation in MDR1 may affect the transport of some classes of substrates but not others (Drescher et al., 2002). It is believed that some of these mutations in Pgp and other ABC transporters may be associated with drug resistance observed in patients with certain types of cancer (Andersen et al., 2009; Drescher et al., 2002).

Nuclear receptors are a group of transcription factors regulated by ligands and can transmit signals from their cognate ligands, leading to substantial alterations in the expression of their target

genes (R. M. Evans & Mangelsdorf, 2014; Sever & Glass, 2013). Nuclear receptors, such as the xenobiotic receptor (SXR), the constitutive androstane receptor (CAR), and some orphan nuclear receptors, have been reported to regulate the induction of the MDR1 gene (Benet & Cummins, 2001; Slosky et al., 2013; Synold et al., 2001; J. Wu et al., 2016). Unlike most nuclear receptors, both CAR and SXR possess unique hydrophobic ligand pockets that are larger and more flexible (Timsit & Negishi, 2007). These xenobiotic-sensing nuclear receptors, SXR and CAR, have been reported to be highly promiscuous and bind to a wide range of exogenous molecules, including drugs and environmental toxicants (G. N. Y. Chan et al., 2013, 2013; Mukherjee & Mani, 2010). Interestingly, several Pgp substrates, such as paclitaxel, rifampicin, and phenobarbital, have been shown to also interact with SXR and CAR (G. N. Y. Chan et al., 2013; Synold et al., 2001) and upregulate the expression of Pgp, as well as some cytochrome P450 isoforms: CYP3A and CYP2C (Chawla et al., 2001; Mukherjee & Mani, 2010; Synold et al., 2001). The correlation between Pgp and the nuclear receptors is highlighted by the increased expression of SXR and CAR in tissues that highly express Pgp, such as the small intestine, liver, kidney, and blood-tissue barriers (Mukherjee & Mani, 2010; Synold et al., 2001). Upon detecting xenobiotics, SXR and CAR upregulate the expression of Pgp, which in turn expels the xenobiotics and frees up the ligand binding sites on the nuclear receptors. This positive feedback loop is believed to drive the clearance of xenobiotics from cells and protects tissues against toxic insults (Chawla et al., 2001). The significant role played by Pgp, SXR, and CAR in the disposition of foreign compounds and overall protection of cells is highlighted by the co-localization of Pgp with SXR and CAR, as well as the increased expression of Pgp in cells exposed to xenobiotics, such as anti-cancer drugs (Cao et al., 2020; Mukherjee & Mani, 2010). Xenobiotics may upregulate Pgp via other mechanisms such the stabilization of the mRNA of MDR1 (Figure 1.2) (Ménez et al., 2012), .

1.2.2 Cellular and subcellular distribution of P-glycoprotein

The synthesis of Pgp occurs in the endoplasmic reticulum (ER), where it is folded before entering the Golgi apparatus for post-translational modifications and maturation (Fu, 2013). During maturation, polysaccharides may be covalently bound to the protein by N-or O-glycosylation to asparagine or serine/threonine residues, respectively. Polysaccharides are long chains of carbohydrates that can be covalently bound to the protein during glycosylation, a process catalyzed by glycosyltransferases (Zabotina et al., 2021). These polysaccharides can be branched or linear and vary in length and composition, depending on the specific glycosylation pathway and the cell type (Zabotina et al., 2021).

Immunofluorescence and confocal studies revealed that Pgp is predominantly associated with the plasma membrane. The protein has also been observed in some intracellular organelles, such as the ER, Golgi apparatus, endosomes, and lysosomes (Fu, 2013). Munteanu et al. (2006), using co-localization of Pgp and green fluorescent protein (GFP), reported the presence of Pgp in the mitochondria (Munteanu et al., 2006). However, subsequent studies revealed that the transporter protein may not be present in the mitochondria (Fu, 2013). Nuclear localization of Pgp has also been reported in doxorubicin-resistant LoVo cell lines (Szaflarski et al., 2013). Unsurprisingly, several reports suggest that localization of Pgp in the ER (synthesis), Golgi (post-translation modifications), endosomes (recycling/trafficking), and lysosomes (degradation) are somewhat transient (Fu, 2013; Molinari et al., 1994). These reports suggest transitory processes such as synthesis, trafficking, and protein recycling occur in these intracellular organelles with the aid of Rab proteins, rather than permanent localization (Figure 1.3). While the mechanism by which Pgp is transported from the ER to the Golgi remains unclear, evidence suggests that Coat Protein II coated (COPII) vesicles and Rab proteins play a significant role in this process (Fu,

2013). The protein is packaged into these COPII vesicles by interacting with specific receptors on the membrane of the vesicles. The protein-containing vesicles bud off the ER and carry the proteins to the Golgi (Fu, 2013). Once the COPII vesicles reach the Golgi, they fuse with the Golgi membrane, and the protein is delivered to the appropriate Golgi compartment for further processing and modification (Fu, 2013). During maturation in the Golgi, the size of Pgp increases from 150 kDa in the ER to 170 kDa in mammals (Molinari et al., 1994). The newly synthesized protein, upon maturation, may be trafficked out of the Golgi to the plasma membrane in one of the two cytoplasmic cytoskeleton-dependent mechanisms (Müsch et al., 1997). The default pathway involves the direct transport of vesicles incorporated with Pgp to the plasma membrane (Fu & Roufogalis, 2007; Kipp & Arias, 2000). Pgp-containing vesicles may also be transported indirectly to the plasma membrane via intracellular endosomes (Fu & Roufogalis, 2007). Studies have shown that Pgp can localize in lysosomes, indicating that lysosomes may play a role in the degradation and recycling of the protein (Sai et al., 1999; Wakabayashi et al., 2005).

1.2.3 Tissue Distribution of P-glycoprotein

Pgp plays a crucial role in several tissues by preventing harmful compounds from entering the circulatory system and aiding in their removal from the blood (Karthika et al., 2022). Previous reports revealed that most mammalian cells express the protein at low levels (Thiebaut et al., 1987). However, Pgp is highly expressed in tissues that are involved in absorption (small intestines and colon), excretion (liver, kidney, pituitary), and the formation of physiological barriers (blood-brain barrier, testis-blood barrier, placenta) (Ambudkar et al., 2003; Sharom, 2007, 2008; Yano et al., 2018). Typically, the transporter facilitates the transport of ligands across cellular lipid membranes, a process essential for various cellular activities, such as nutrient uptake, waste elimination, energy generation, and cell signaling.

1.2.4 P-glycoprotein Expression in the Gastrointestinal System

Xenobiotics are mainly absorbed in the small intestine after oral administration. In mammals, the small intestine mainly absorbs nutrients through passive diffusion (simple or facilitated) or active diffusion via influx transporters (L. M. Chan et al., 2004; Kiela & Ghishan, 2016; Takano et al., 2006). The apical surface of the small intestine is covered with specialized microscopic cellular membrane protrusions known as microvilli, which greatly enhance the absorptive capacity of the small intestine by increasing its surface area (Walton et al., 2016). Most drugs are administered orally for convenience and safety (Stewart et al., 2016). The high expression of efflux transporters on the microvilli of the small and large intestines acts as the first line of defense against the high influx of xenobiotics that could potentially enter the cells through passive diffusion. (Takano et al., 2006).

Pgp expression levels vary along the gastrointestinal (GI) tract, with the stomach showing the lowest and the colon showing the highest (Leslie et al., 2005; Takano et al., 2006; Y. Zhang & Benet, 2001) which makes sense given that absorption compounds occurs highest in the small intestines. Pgp localized on the apical surface of small intestinal enterocytes expels xenobiotics back into the intestinal lumen, limiting the absorption of these compounds (Figure 1.4A). Although most drugs are absorbed in the small intestine, Biopharmaceutics Classification System (BCS) class I drugs are primarily absorbed in the colon, with a relative bioavailability greater than 70% (Yihong Qui et al., 2016). In the colon, Pgp expression is higher in the mucosal layer, which lines the inside of the colon, compared to the muscular layer (Foley et al., 2021). This observation suggests that P-gp may play a role in protecting the colon from harmful substances that may be present in the gut lumen. Studies have also shown that P-gp expression in the colon can be influenced by various factors, including diet, gender, species, and pathological state (Foley et al.,

2021; Linn & Giaccone, 1995; Mai et al., 2021). Thus, the presence of Pgp in the gastrointestinal tract may serve as a barrier to oral xenobiotics, hindering the passive diffusion of substances across the small and large intestines.

1.2.5 P-glycoprotein Expression in Hepatocytes

Xenobiotics absorbed from the intestines are transported to the liver via the portal vein, where they passively diffuse into the hepatocytes. Once in the liver, most of these compounds undergo metabolism before entering the systemic circulation (L. M. Chan et al., 2004; Leslie et al., 2005). Unsurprisingly, the liver has the highest expression of both phase I and II metabolizing enzymes (Almazroo et al., 2017; Drozdik et al., 2018). In addition, many metabolizing enzymes share substrate specificity with ABC efflux transporters, which create an effective coupled mechanism that facilitates the clearance of compounds, such as drugs and toxins, from the liver (Benet & Cummins, 2001; L. M. Chan et al., 2004; Suzuki & Sugiyama, 2000). ABC transporters, including Pgp, are primarily located on the canalicular membrane of hepatocytes (Figure 1.4B) (Ben Saad et al., 2021). These transporter proteins extrude xenobiotics and their metabolized product into the bile duct as bile salt, which is then returned to the intestinal lumen for excretion (Ben Saad et al., 2021; Wakabayashi et al., 2006). Thus, by acting as 'safety guards,' Pgp and other ABC transporters localized in hepatocytes excrete plasma-bound compounds that initially escaped the stomach and intestines (GI) back into the intestinal lumen via bile salts, from where they are eventually eliminated from the body (Annaert et al., 2001; Kong et al., 2016; Leslie et al., 2005). The expression of Pgp in the liver can be influenced by several factors, including age, diet, gender, genetic polymorphisms, and exposure to various drugs and toxins (Ahmed Juvalé et al., 2022; Foucaud-Vignault et al., 2011; Sun et al., 2021; Thornton et al., 2008). For example, some drugs, such as rifampin, carbamazepine, and phenobarbital, can induce the expression of Pgp in the liver,

leading to increased efflux of other drugs that are substrates of the transporter (Asaumi et al., 2022; Finch & Pillans, 2014).

1.2.6 Renal Expression and Function of P-glycoprotein

After xenobiotics and their metabolites enter the circulatory system from the liver, they are distributed throughout the body (Edwards & Wanless, 2013; R. Li et al., 2016). The systemic distribution of xenobiotics is influenced by several factors, such as their chemical properties, the state of the circulatory system, and the activity of various transporters (Mao et al., 2018). The kidneys act as a filtration system and are crucial in removing metabolized or unaltered xenobiotics from the blood as components of urine (Yin & Wang, 2016). The proximal tubules are responsible for reabsorbing essential molecules and ions from the filtrate produced by the glomeruli (Lea-Henry et al., 2018). However, re-absorption of potentially harmful compounds back into the body is possible. Pgp is highly expressed in the proximal tubule cells' apical membrane, where it efflux various xenobiotics, including drugs, toxins, and metabolites back into the urine (Peeters et al., 2011) (Figure 1.4C). Thus, the high localization of Pgp prevents the re-absorption of potentially harmful compounds by transporting them back into the lumen of the nephron for excretion. This process ensures the removal of xenobiotics from the body as component of urine. P-glycoprotein is also expressed in the thick loop of Henle and collecting duct of the kidney, where further re-absorption of essential components of the glomerular filtrate occurs (Basit et al., 2019; Ernest & Bello-Reuss, 1998; Zacchia et al., 2018). Studies with Mdr1a/Mdr1b mice showed that the renal clearance of xenobiotics, such as vinblastine and arsenic, was significantly reduced in Mdr1 knockout mice compared to wild-type mice (J. Liu et al., 2002; Schinkel et al., 1995). Pgp's ability to eliminate xenobiotics via urine helps to maintain renal homeostasis and prevent the accumulation of harmful toxins in the body.

1.2.7 Expression and Function of P-glycoprotein at Physiological Barriers

P-glycoprotein (Pgp) is present at several physiological barriers, including the blood-brain barrier (BBB), the placental barrier, and the blood-barrier barrier.

1.2.7.1 The blood-brain barrier

The blood-brain barrier (BBB) is a network of blood vessels and tissues made of continuous endothelial cells with a tight junction coupled with transporter proteins that protect neurons in the central nervous system (CNS) (Abbott, 2014; Y. Chen & Liu, 2012). The expression of Pgp on the apical surface of endothelial cells at the BBB (Figure 1.5D) safeguards the brain from toxic substances (De Lange et al., 2018) by preventing potentially harmful compounds from entering the parenchymal cells of the brain (Aryal et al., 2017). The importance of Pgp at the BBB is highlighted by the observed correlation between the limited development of Pgp and the heightened sensitivity of neonates to the central depressive effects of opioids (Lam et al., 2015). The function of the tight junction at the BBB is limited in preventing the entry of hydrophobic and amphiphilic xenobiotics. Hence, the role of Pgp and other ABC transporters expressed at the barrier becomes crucial in preventing the entry of such compounds into the CNS (Tachikawa et al., 2014; Zlokovic, 2008). *In vivo* studies have shown that Mdr1a/b(-/-) knockout mice exhibit a higher sensitivity (3-100 fold) to neurotoxicity caused by Pgp-substrates compared to wild-type mice (Bauer et al., 2019; Lankas et al., 1997; Schinkel et al., 1994). The absence of Pgp in knockout mice resulted in approximately two-fold higher plasma concentrations of digoxin compared to Pgp-expressing mice. The knockout mice had approximately 35-fold higher brain digoxin concentrations than the control animals (Fromm, 2004; Schinkel et al., 1995). Other studies have also highlighted the importance of Pgp at the BBB in protecting neural tissues from potentially toxic xenobiotics and revealed the severe consequences of eliminating Pgp from these

tissues (Agarwal et al., 2012; Löscher & Gericke, 2020; Tachikawa et al., 2014). The ability of Pgp to eliminate xenobiotics at the BBB, along with the physical barrier of the junction, collectively diminishes the accumulation of compounds in the CNS, safeguard the CNS, and upholds neuronal homeostasis

1.2.3.2 The blood placenta barrier

The placenta is a physical and immunological barrier that regulates the hematogenous exchange of endogenous and exogenous substances between the maternal and fetal cells (Megli & Coyne, 2022). The blood placenta barrier (BPB) consists of a single-layered syncytium of polarized trophoblast cells and the fetal capillary endothelium separating maternal and fetal circulation (Blundell et al., 2016). This blood-tissue barrier protects the fetus from exposure to potentially harmful xenobiotics (Leslie et al., 2005), which is critical in maintaining homeostasis in the placenta and for the healthy development of the fetus. The apical membrane of the syncytiotrophoblast (maternal-facing side of the layer) is exposed to maternal blood, while the basolateral (fetus-facing side of the membrane) is in contact with the cytotrophoblast and fetal blood vessels (Leslie et al., 2005). Several active membrane transporters, such as Pgp (Atkinson et al., 2003; Ushigome et al., 2000), BCRP (Grube et al., 2007), MRP2 (Schwabedissen et al., 2005; St-Pierre et al., 2000), and MRP3 (St-Pierre et al., 2000) are expressed in both the apical and basolateral side of the syncytiotrophoblast (Atkinson et al., 2003; Grube et al., 2007; Ushigome et al., 2000). However, the functional direction transport (efflux or influx) for most of the ABC transporters on the basolateral side of the syncytiotrophoblast has not been fully established. Breast Cancer Resistance Protein (BCRP) and Pgp are two of the most highly expressed ABC transporters on the placenta's basolateral and apical surface syncytiotrophoblast layer (Figure 1.4E). *In vivo*, evidence of the role Pgp plays in protecting fetal development is

highlighted by the development of cleft palate in the offspring of Mdr1a/b(-/-) mice treated with avermectin. Whereas the wild-type did not show any observable abnormalities, Mdr1a/b(-/+) mice showed increased sensitivity to avermectin (Lankas et al., 1997). The protective role Pgp plays in the BPB for the fetus has been reported in other animal studies with genetically modified models (Lankas et al., 1998) or by chemical inhibition of Pgp (Bhuiyan et al., 2012; Eyal et al., 2009). In these studies, an increased accumulation of potentially harmful substances in fetal tissue was observed compared to the control. These studies highlight the significance of placental Pgp in safeguarding the fetus against heightened vulnerability to potentially harmful substances, which can result in congenital disabilities, abnormalities, and even mortality.

1.2.3.3 The blood-testis barrier

Blood-testis barrier (BTB) is a tight blood-tissue barrier in mammals that creates an immune-protected site for post-meiotic spermatid development. Besides protecting maturing gametes from the production of antibodies against spermatid-specific antigens, BTB also defends gametes against the exposure of potentially harmful xenobiotics and other endogenous compounds (Su et al., 2011). Pgp is highly expressed on the apical surface of the capillary endothelium cells of the testis (Choo et al., 2000; Schinkel et al., 1994; Su et al., 2009). Aside from the capillary, Pgp has also been observed in Leydig cells, testicular macrophages, peritubular cells, Sertoli cells, and haploid spermatozoa (Melaine et al., 2002; Su et al., 2013). Particularly important is the expression of the Pgp on the basal surface of the Sertoli cells. Like BBB, BTB is characterized by continuous epithelial Sertoli cells joined by tight junctions (Hau et al., 2023; Leslie et al., 2005). The expression of Pgp on the basal surface of the Sertoli cells protect accumulation of potentially toxic compounds from accumulating in the adluminal/apicolateral and preserve the normal physiological function of the testes (Hau et al., 2023) (Figure 1.4E). Mdr1a/b(-/-) knockout mice

had an increased accumulation of Pgp substrates within testicular tissue compared to the control (Choo et al., 2000; Schinkel et al., 1994). About a 4-fold increase in the accumulation of Pgp substrates was observed when wild-type mice were treated with nelfinavir, a potent Pgp inhibitor (Choo et al., 2000). Thus, Pgp expression at the BTB primarily protects developing gametes from potentially harmful substances and ensures the health of future offspring.

1.2.3.4 P-glycoprotein Expression in Tumor Cells

Many cancer cells acquire resistance to a wide range of structurally diverse chemotherapeutic agents, resulting in the acquisition of MDR. Whereas some cancer cells, such as renal and melanoma tumors, have been observed to have a higher constitutive expression of P-gp (Henrique et al., 2013), an induced expression of Pgp as a defense mechanism has been observed in most solid tumors (Amawi et al., 2019). The overexpression of Pgp in cancer cells can occur through one or a combination of two or more mechanisms. The main mechanism observed in the upregulation of Pgp in tumor cells involves the alterations in the regulatory gene that encodes the transporter, the MDR1 gene. One way this can happen is through gene amplification, where the cancer cells develop multiple copies of the MDR1 gene (K. G. Chen et al., 2002; Genovese et al., 2017; Robinson & Tiriveedhi, 2020). This phenomenon may lead to an increase in the amount of Pgp produced, leading to the development of MDR. Mutations in the MDR1 gene can lead to the production of highly functionalized proteins by tumor cells. These mutations can lead to changes in the structure and function of the protein, which can make the transporter more effective at extruding drugs out of cells (Ambudkar et al., 2003; Fung et al., 2014). Thirdly, epigenetic modifications, such as DNA methylation and histone acetylation, can affect the accessibility of the MDR1 genes to transcription factors and other regulatory proteins. An increased rate of epigenetic modifications in cancer cells in comparison to control has been reported in several studies (Baylin

& Jones, 2016; L.-C. Li et al., 2005). Epigenetic changes in the genes that transcript regulatory and other related proteins that modulate the Abcb1 gene expression in cancer cells may also influence the expression of the Pgp (Henrique et al., 2013; Zappe & Cichna-Markl, 2020). The activation of some transcription factors, such as the NF- κ B and AP-1 (Q. Chen et al., 2014; Prajoko & Aryandono, 2019), and stimulation of signaling pathways, such as PI3K/Akt/mTOR pathways, that are usually activated in cancer cells may increase the expression of Pgp (Association et al., 2022; Dong et al., 2021; R. Liu et al., 2020). Post-transcriptional miRNA regulations, where miRNAs, such as miR-145 and miR-331-5p, bind to the 3' untranslated region of MDR1 mRNA to decrease the protein translation, can also influence the amount of Pgp produced. Tumor micro-environments, including hypoxia and inflammation, can also influence Pgp expression in cancer cells (Z. Chen et al., 2023; Emami Nejad et al., 2021; X. Jing et al., 2019; Roy et al., 2020). For instance, hypoxia can upregulate the expression of Pgp via Hypoxia-inducible Factor (HF-1 α) that binds to the MDR1 gene and upregulate the protein (Bossennec et al., 2018). Overall, the overexpression of Pgp in cancer cells can occur through various mechanisms. Understanding these mechanisms is vital for developing effective strategies to multi-drug resistance in chemotherapy.

1.2.3.5 P-glycoprotein Expression in immune cells

Over the past decade, several studies have highlighted the expression of Pgp in many types of immune cells, where they play a critical role in protecting these cells from toxic compounds and drugs (Bossennec et al., 2018; M. L. Chen et al., 2020; Kooij et al., 2009; Pendse et al., 2003). Both human and animal T-cells express Pgp. It is believed that Pgp influences physiological processes such as cell migration, differentiation, survival, and cytotoxic functions (Pilotto Heming et al., 2022). Both molecular and cellular analyses of Pgp showed a high expression of the protein in some immune cells, such as natural killer (NK) and B-lymphocytes (Egashira et al., 1999;

Robinson & Tiriveedhi, 2020). The transporter is present at the membrane of specific subsets of T-cells (CD4⁺ and CD8⁺) that secrete and express CD16 and IL-17A, respectively (Bossennec et al., 2018). Pgp overexpression has also been noted in resident dendritic cells and macrophages but not monocytes (Bossennec et al., 2018). Conversely, monocytes are believed to highly express multi-drug resistance protein 1 (MRP1) instead of Pgp (Kruh & Belinsky, 2003). Kooij et al. observed that Pgp inhibition could enhance T cell proliferation and cytokine production (Kooij et al., 2009). In vivo studies involving Mdr1a^{-/-} and control mice reported that high levels of Pgp expression and activity appear necessary for some lymphocytes' normal development (Eisenbraun et al., 2000). In fact, changes in the function and levels of Pgp in immune cells have been associated with several diseases, such as auto-immune disease, Crohn's disease, HIV treatment resistance, and other immune-associated pathologies (Chaillou et al., 2002; Ramesh et al., 2014; Tsujimura et al., 2008).

1.2.3.6 The Role of P-glycoprotein in Plants

In plants, ABC transporters have been shown to regulate the uptake and transport of various molecules, such as nutrients, toxins, and hormones. Some ABC transporters protect plants from toxins and other harmful environmental substances and can confer drug resistance on the plants (Jasiński et al., 2001). The importance of ABC transporters to plants is highlighted by the relatively large number of putative ABC transporter genes (>120 members) in the genome of Arabidopsis when compared to other multicellular organisms of comparable genome size (usually about 50 ABC members) (Geisler & Murphy, 2006). A significant number of ABC transporters have also been identified in other plants, such as rice and barley (Davies et al., 1997; Geisler & Murphy, 2006). Most of the ABC transporters characterized in plants are found in the vacuolar membrane and are believed to be involved in the intracellular sequestration of cytotoxins (Jasiński et al.,

2001). Pgp homologs in plants can act as an efflux pump, pumping these substances out of the plant cells and preventing them from accumulating to toxic levels (Martinoia et al., 2002), which is particularly important for plants growing in contaminated soils or other polluted environments. Pgp homologs in plants have also been shown to be involved in transporting various hormones, such as auxins (Blakeslee et al., 2007) and gibberellins (Fujioka et al., 1997). Thus, P-gp can assist in regulating the distribution and concentration of plant hormones, which are essential for plant growth and development. For instance, in *Arabidopsis thaliana*, mutations in Pgp homologs altered the auxin distribution, resulting in developmental defects (Blakeslee et al., 2007). Fujioka et al. (1997) reported that mutation of Pgp homologs in rice resulted in adverse effects, such as dwarfism and reduced seed germination (Fujioka et al., 1997). Additionally, Pgp homologs in plants have been implicated in regulating the uptake of nutrients, such as iron, zinc, and copper (Mills et al., 2005). Therefore, the expression of Pgp analogs can be important for plants growing in nutrient-deficient soils or for plants exposed to high levels of these metals, which can be toxic at high concentrations. Overall, Pgp analogs play an essential role in regulating the transport of various molecules in plants, including cytotoxins, hormones, and nutrients; disruptions to the function of Pgp homologs in plants can have significant consequences on their growth and development.

1.3 THE ARCHITECTURE OF P-GLYCOPROTEIN

Pgp has been reported to render most therapeutic agents ineffective by preventing them from reaching their target tissues. To better understand Pgp-mediated transport and improve drug design and development, it is imperative to understand the atomic structures of the transporter protein. The ABC transporters are characterized by transmembrane helices (TM) spanning the membrane and nucleotide-binding domains (NBDs) that bind and hydrolyze ATP. The transmembrane domain (TMD) often determines the substrate specificity for this superfamily of

proteins (Yu et al., 2015). In fact, the number of TMs and overall fold often dictate the structure and specificity of ABC transporters within the superfamily (Schinkel & Jonker, 2003; Srikant et al., 2020). Either as an exporter or importer of solutes, the core architecture of the ABC superfamily consists of two TMDs and two NBDs (Wilkins, 2015). Some ABC transporters are realized as a complex of two or more proteins fused together, whereas others are single polypeptides with all four domains present (Davidson et al., 2008; Schmitt & Tampé, 2002). Common to all ABC family members, transport is powered by ATP hydrolysis, which results in significant conformational changes within the TMD (Loo et al., 2010, 2012). Many cancer cells overexpress ABC efflux transporters as a primary mechanism of therapeutic resistance. ABC efflux extrudes important chemotherapeutic agents out of cells, even against the concentration gradient, and prevents the accumulation of drugs to therapeutically relevant levels in the target tissues. To better understand this role in resistance, Victor Ling and his group extracted a large glycoprotein from the membrane of colchicine-resistant Chinese hamster ovarian cells (Juliano & Ling, 1976). This multi-drug resistant protein was identified as a 170 kDa transmembrane protein called P-glycoprotein (Pgp). (Juliano & Ling, 1976) After its discovery, Pgp was determined to be expressed ubiquitously in many of the mammalian cell lines used in in vitro assays (Sharom, 2011b). Among the superfamily of ABC transporter, Pgp is the most studied (Robinson & Tiriveedhi, 2020). The structure of Pgp consists of two identical halves, each consisting of six membrane-spanning domains fused with a cytoplasmic domain.

1.3.1 High-Resolution atomic models of P-glycoprotein

Atomic models and density maps of Pgp with and without ligands, determined using x-ray and cryo-EM methods, have been submitted to the Protein Databank (PDB) and Electron Microscopy Databank (EMDB). These available atomic structures of Pgp provide immense detail

on the mechanism of substrate recognition, ATP hydrolysis, drug transport, and inhibition. Across all species, the murine Pgp (Mdr1a) or *mPgp* has been the most well-studied. The *mPgp* has about 87% sequence identity to human Pgp (*hPgp*, ABCB1), which makes it an excellent model for probing the structural mechanism underlying Pgp-mediated transport in humans. In addition, isolated *mPgp*, compared to the *hPgp*, has been more stable and can be expressed in several host organisms, including yeast (Waghray & Zhang, 2017). The atomic structure of *mPgp* at high resolution has been solved in several studies (Aller et al., 2009; Barbieri et al., 2021; Esser et al., 2017; Kim & Chen, 2018; J. Li et al., 2014; A. B. Ward et al., 2013). However, until recently, no human or murine ABCB1 protein structures bound to substrates were available. Most of the first solved *mPgp* structures were determined in the absence of ligands (Figure 1.5B, PDB ID 4M1M) (Aller et al., 2009) due to the relatively high stability of the protein in an unbound state (Kodan et al., 2019). Structural analysis of *mPgp* revealed an inward-facing conformation with a large binding cavity that is exposed to the lipid membrane and the NBDs set apart (Figure 1.5A, 1.5B, and 1.5C) (Esser et al., 2017; J. Li et al., 2014; A. B. Ward et al., 2013). Earlier structural analysis and experimental data showed that the binding cavity of *mPgp* has an internal volume of 6,000 Å³, with the NBDs separated by a distance of 30Å (Aller et al., 2009; Eckford & Sharom, 2009). The large Pgp binding cavity is believed to allow the protein to bind to structurally diverse substrates ranging from small molecules, such as Bisphenol A (Speidel et al., 2018), to large peptides, like amyloid-β (Cirrito et al., 2005). The above reasoning also accounts for the protein's ability to bind to multiple substrates simultaneously (Aller et al., 2009). Further analyses of the binding cavity reveal the absence of polar residues within this cavity (J. Li et al., 2014). Unlike the *mPgp*, bacterial lipid flippase MsbA and Pgp homolog, MDR3 (ABCB4) (J. Li et al., 2014) showed significant polar residues in their binding cavity. With the above data, we hypothesize that the hydrophobic

nature of the *mPgp* and *hPgp* binding cavities underlies the ability of the transporter to efflux a wide range of hydrophobic and amphiphilic xenobiotics. The limited number of polar residues at the binding cavity may have also accounted for the inability of the Pgp to act as a lipid flippase. The first high-resolution protein structure of *hPgp* was recently solved with a resolution of 3.4 Å by Youngjin Kim and Jue Chen (2018) in a D-dodecyl-β-maltoside (DDM) detergent micelle using cryo-EM (Figure 1.3B, PDB ID 6C0V) (Kim & Chen, 2018). To achieve this structure, the authors mutated *hPgp* in both nucleotide-binding domains (E556Q/E1201Q) to make a catalytically inactive transporter, which was then fused with a GFP tag for expression and purification (Kim & Chen, 2018). The mutations at the NBDs were done to prevent ATP hydrolysis. The structure was then solved in the presence of saturating ATP and vinblastine (a known Pgp substrate) with an attempt to trap the protein in a ligand-bound conformation. The authors of this study observed one molecule of ATP trapped between the dimerized NBDs in their electron density mapping (Kim & Chen, 2018). The absence of vinblastine coupled with the collapse of the drug-binding cavity suggests that the resolved conformation *hPgp* observed is likely a post-translocation state (when the substrate had been released to the extracellular state but before ATP hydrolysis). This groundbreaking study reported for the first time a solved structure of human Pgp in an outward-facing conformation that correlates with ATP-induced conformation of the transporter protein. Alam et al.(2019) recently reported the first near-atomic resolution (3.5 Å) cryo-EM structures of human Pgp re-constituted in lipid and cholesterol, complexed with Taxol (transporter substrate) or zosuquidar (Pgp inhibitor) (Figure 1.3) (Alam et al., 2019). This study is significant, as it accounted for the possible effect of the lipid bilayer on activity of the transporter protein. Prior to this study, the crystal structure of Pgp had been determined only with detergent-solubilized protein without

considering the unique role the lipids play in regulating the conformation and function of the protein (Sharom, 2014, p.).

1.3.2 Modulating Role of Lipid in Pgp-mediated Transport

The function of Pgp is highly influenced by the fluidity of the membrane (Sharom, 2014). The lipid shell composition surrounding a protein differs depending on the specific protein (Corradi et al., 2019). Previous studies revealed that lipid compositions of extracted membranes around Pgp are enriched with phosphatidylethanolamine and phosphatidylserine (Sharom, 2014). Transmembrane proteins are directly in contact with the lipid membrane, forming unique lipid-protein interactions (Corradi et al., 2018). An electron paramagnetic resonance spectroscopy study observed restricted mobility of lipids that interact with integral membrane proteins (Fyfe et al., 2001). Membrane proteins present multiple grooves and clefts on the surface of membranes, resulting in the distortion of lipids that fill the space around the protein (Fyfe et al., 2001). This intimate lipid-protein interaction plays a substantial role in the structure and function of membrane-embedded proteins (Tran et al., 2023). Thus, the lipid composition surrounding Pgp may play a significant role in substrate binding and transport. It has been observed in earlier studies that ATPase activity is absent for detergent-solubilized Pgp unless the protein is embedded in and interacting with lipids, suggesting that the lipid environment is critical for substrate permeability, binding, and transport (Sharom, 2014). In addition, earlier studies have shown that changes in the lipid composition of the membrane can alter the activity and substrate specificity of Pgp. For instance, increasing the level of cholesterol and phosphatidylcholine in the membrane influenced the activity of P-gp (Garrigues et al., 2002; Simon & Schubert, 2012; Szlasa et al., 2020; Tran et al., 2023). Decreasing cholesterol levels in a cell membrane have been shown to reduce Pgp-mediated ATPase activity (Kimura et al., 2007; Kodan et al., 2009). Phospholipids and

sphingolipids, two of the major components of the plasma membrane, also interact with transmembrane domains of Pgp (Radeva et al., 2005). It is worth noting that P-gp is primarily known for its role in drug efflux, and increased Pgp activity can contribute to drug resistance in cancer cells. Therefore, any potential interaction between membrane lipids and Pgp activity may have implications for drug development and treatment strategies. Unfortunately, the mechanism of membrane lipid composition affecting Pgp activity is not fully understood. It is possible that the membrane lipids affect the expression of genes encoding Pgp or interact directly with the protein to enhance its function. Even though, plethora of evidence available support the later theory, more research is needed to fully understand this relationship and inform rational drug design aimed at circumventing the actions of the transporter.

1.4 P-GLYCOPROTEIN-MEDIATED SUBSTRATE TRANSLOCATION MODELS

The substrate binding and transport models of Pgp are complex, and several hypotheses have been proposed to explain these processes. Pgp is a membrane-bound protein, with its extracellular component protruding approximately 25 Å from the membrane. At the same time, the NBD is believed to extend around 75 Å into the cytosolic region (Sigdel et al., 2018)(Fig. 1.4). Even though the exact mechanism of substrate binding and subsequent transport of substrate by the protein is yet to be resolved, there are currently three widely accepted models. The orientation of the transporter allows the lipid membrane to make direct contact with the relatively large hydrophobic cavity, suggesting that xenobiotics may enter the cavity through the plasma membrane. The available models include substrate flippase, alternating access, and the hydrophobic vacuum cleaner (Sharom, 2011b, 2014). It is important to note that these models are not the only proposed mechanisms for Pgp substrate binding and transport (Hennessy & Spiers, 2007). Numerous laboratories, including ours, are currently conducting research to gain a comprehensive understanding of the intricate mechanisms of this critical transporter.

1.4.1 The hydrophobic vacuum cleaner model

The hydrophobic vacuum cleaner model proposes that xenobiotics would be "sucked" into the binding cavity of the protein prior to their translocation by Pgp. Upon binding and conformational rearrangement, the transporter would expel xenobiotics back to the extracellular environment, irrespective of the concentration gradient. The hydrophobic vacuum cleaner model is perhaps the most accepted model for the substrate binding and transport mechanism of Pgp. According to this model, Pgp has multiple binding sites for hydrophobic compounds, which include the lipid bilayer, a large central cavity, and two drug-binding pockets (Sharom, 2014). These sites work together to transport substrates across the membrane. The central cavity of the

transporter is believed to function as a hydrophobic chamber that can accommodate a wide range of substrates. The two drug-binding pockets are located at the interface between the transmembrane and nucleotide-binding domains. These pockets are thought to be the primary binding sites for drugs and other hydrophobic substrates. In this model, Pgp transports substrates by a combination of passive diffusion and active transport. The primarily hydrophobic substrates first enter the lipid bilayer and then are transferred to the central cavity of Pgp. Once inside the central cavity, the substrate can interact with the drug-binding pockets, triggering conformational changes. The conformational changes in protein may lead to substrate translocation across the membrane. This model is supported by various experimental studies, including X-ray crystallography; electron microscopy; and biochemical and biophysical assays, which have shown that P-gp can interact with substrates in multiple ways, including binding to the lipid bilayer and forming transient hydrophobic interactions with substrates (Hennessy & Spiers, 2007; J. Li et al., 2014; Sharom, 2014). For instance, data available from the solved structure of *hPgp* and *mPgp* show that the lateral opening formed by helices 4,6 and 10,12 is exposed to the outer membrane leaflet in the unbound state of *mPgp* structure (Pomorski et al., 2004)) and maintain this posture upon conformational rearrangement of the protein in which the shift the protein shift to the outward-facing orientation as shown by the structure *hPgp* (Kim & Chen, 2018)). Even as the transporter moves from one conformation to the other, continuous closure to the outer leaflet suggests that compounds are expelled back into the lumen, not into the inter-leaflet space of the membrane.

1.4.2 The Substrate Flippase Model

The substrate flippase model for Pgp transport of substrate was proposed due to the sequence similarity to MDR3, which acts as a lipid flippase (Pomorski et al., 2004). Studies

suggest that Pgp in an unbound state has the NBDs far apart, with the binding cavity in the TMDs having access to the inner leaflet of the membrane (Esser et al., 2017; J. Li et al., 2014; A. B. Ward et al., 2013). In the substrate flippase model, xenobiotics enter the binding cavity of Pgp through the inner leaflet of the membrane (Hennessy & Spiers, 2007; Sharom, 2011b, 2014), which may result in conformational changes in the transporter. These conformational rearrangements in the protein increase the accessibility of the outer leaflet. Subsequently, the substrates diffuse back into the outer leaflet membrane along the concentration gradient within the membrane, not across the membrane like the hydrophobic vacuum model (Sharom, 2011b, 2014).

1.4.3 Alternating access

The alternating access model attempts to explain the mechanism mediating the coupling of ATP hydrolysis to the transport of substrates by ABC transporters. In the alternating access model, Pgp in the apo state has an inward-facing conformation where the substrate-binding site is exposed to the cytoplasm, allowing substrates to bind. Following the binding of the substrate, the transporter undergoes a conformational change, which results in an outward-facing orientation. The outward-facing orientation exposes the substrate-binding site to the extracellular environment, releasing the substrate. The energy required for driving the transporter from the inward-facing to the outward-facing conformation is provided by the binding and subsequent hydrolysis of ATP molecules. Several structural studies have revealed that Pgp exhibits several conformations dependent on the presence of substrates and ATP, which support this model (Kerr et al., 2001; Sharom, 2006; Siarheyeva et al., 2010).

1.4.3. Alternating binding model for P-glycoprotein-mediated ATP hydrolysis

Despite several decades of investigations and technological advancements, the exact mechanism by which ATP is coupled to the transporter remains elusive, which is surprising.

However, biochemical data analyses suggest that coupling ATP hydrolysis to Pgp-mediated transport may occur through the alternating site model. The alternating site model proposes that the binding of ATP to Pgp occurs in stepwise alternating manner, sandwiched by drug binding. This model infers that the catalytic cycle of Pgp begins with an ATP binding a NBDs. The second ATP molecule binds after substrate binds, which followed by the hydrolysis of an ATP molecule and the release of an inorganic phosphate. These series of events drive the conformational changes required to release the substrate on the opposite side of the membrane. The release of the bound ADP molecule returns the protein to the apo inward-facing conformation. For this mechanism to occur, one ATP binding site must have a higher affinity in the apo inward-facing state, and the affinity for these sites must switch as the transport cycle continues. The alternating binding model is supported by some biochemical and structural data. For instance, during the Pgp catalytic cycle, the binding of ATP at the NBDs results in the formation of the ADP-Pi-Mg²⁺ complex. Vanadate-promoted photooxidative cleavage has shown that ATP hydrolysis occurs in one or the other ATP site but not both simultaneously (Esser et al., 2017). Therefore, it appears that only one NBD is catalytically active while the other NBD is in the transition state. Further, the analyses of ATP-bound *h*Pgp structure show that the binding of both ATP molecules is required to form the NBD sandwich (Kim & Chen, 2018). In fact, Pgp equipped with a single active NBD lacked catalytic activity. For instance, X-ray crystallography solved a catalytically inactive *m*Pgp in the presence of saturating ATP at 3.35 Å resolution (Esser et al., 2017). In this study, only one ATP molecule was bound in NBD1 in the absence of Pgp substrates, and the protein remained in the inward-facing conformation, akin to apo Pgp. This study also demonstrated that, in the apo state, the affinity of ATP at each NBD is different. However, it remains unclear why ATP molecules did not occupy both NBDs as observed in the *h*Pgp structure at saturating concentrations. In their attempt

to improve the crystallization and structure resolution of the *mPgp* transporter, Esser et al. (2017) reduced the protein dynamics by shortening the loop that connects the two halves of the protein at the NBD (Esser et al., 2017). This loop is believed to play a role in communicating the affinity switch between the NBDs (Dehghani-Ghahnaviyeh et al., 2021). Shortening of the loop decreased the transporter's ATPase activity, indicated by the binding of only one ATP molecule to the NBD1 (Esser et al., 2017). However, the asymmetric ATP bound conformation was observed, which supports the asymmetric catalytic cycle model (Esser et al., 2017). In this conformation, one ATP molecule binds tightly to the NBD, while the other binds loosely to the other NBD, causing the transporter to shift into a closed conformation (Esser et al., 2017). It is still unknown whether one or two hydrolysis events are responsible for bringing the transporter back to the open conformation, and further research is needed to resolve this issue. The *hPgp* structure provides further evidence for this model by showing that the second ATP molecule binds loosely to the other NBD and shifts the transporter into an outward-facing conformation (Kim & Chen, 2018). Orthovanadate (Vi) trapping has been used to study protein conformational studies in the protein. In these biochemical studies, ADP-Vi-Mg²⁺ complexes non-reversibly formed trap the transporter in an ATP-bound state, instead of the ADP-Pi-Mg²⁺ complexes that readily breaks down to release the Pi (Sharom, 2006, 2011b; Siarheyeva et al., 2010). Furthermore, when only one NBD was trapped in the ADP-Vi-Mg²⁺ complex, the activity of the protein was abolished, which suggests both NBDs must be active to couple the transport of substrates (Sharom, 2006, 2011b; Siarheyeva et al., 2010).

1.4.4 Proposed ATP coupled P-glycoprotein catalytic cycle: from ligand binding to crosstalk between TMD and NBD

P-glycoprotein has significant basal ATPase activity even in the absence of substrates (Loo et al., 2012). Interestingly, substrates and inhibitors of Pgp may stimulate, inhibit, or have no effect on the basal ATPase activity of the protein upon binding (Loo et al., 2012; Nervi et al., 2010) (Ledwitch et al., 2016; Loo & Clarke, 2014b; Wilt et al., 2017,). For instance, whereas Hoechst 33342 (Pgp substrate) maintains ATPase basal activity, tariquidar (Pgp inhibitor) has > 2-fold stimulatory effect on Pgp-mediated ATPase activity (Clay & Sharom, 2013; Loo & Clarke, 2014c). On the other hand, verapamil may stimulate or inhibit Pgp-mediated ATPase activity depending on the prevailing concentration of the drug (Ledwitch, Barnes, et al., 2016; Loo & Clarke, 2014c). Available literature suggests that Pgp has at least three substrate-binding sites. These include the R- and H-binding sites and a third binding site that exerts an allosteric influence on the R and H sites. In this context, the terms R and H refer to the specific binding sites of Rhodamine 123 and Hoechst 33342, respectively, on Pgp (Chufan et al., 2013). In addition, ligand binding, epitope accessibility, spectroscopic, protease susceptibility, and other biochemical studies suggest that the TM undergoes a significant shift in orientation upon the binding of nucleotides (Safa, 2004). In fact, it is believed that nucleotide binding, rather than hydrolysis drives the conformational changes required for the successful translocation of substrates by the transporter protein (Kim & Chen, 2018; Safa, 2004). The substrate binding to Pgp induces conformational rearrangements that reduce the distance between the two NBDs, promoting ATP binding and hydrolysis (Zoghbi et al., 2017). Finally, dissociation of the substrate, coupled with ATP hydrolysis and ADP dissociation, reset the protein to the inward-facing conformation. For the necessary conformational changes in the TMD to occur, the changes in the orientation associated with the binding of ATP must translate

to the NBDs. Extracellular loops (ECLs) and intracellular loops (ICLS) in each half of the Pgp connect the transmembrane helices (Figure 1.7). The significance of these loops is highlighted by their contribution to the functionality of Pgp, which include various processes such as protein folding, recognizing substrates, hydrolyzing ATP, and facilitating the transport of drugs across the membrane. The crosstalk between the two domains on each half of the transporter has been hypothesized to be intermediated by the ICLs of the transporter (Loo & Clarke, 2014b). These loops are essential for the function of Pgp, as they play a role in proper protein folding, substrate recognition, ATP hydrolysis, and conformational changes that facilitate the transport of drugs across the membrane (Loo & Clarke, 2014a). The ICLs also connect the TMs to the NBDs, a common structural feature in most ABC transporters. In Figure 1.7, each ICL and ECL loop is identified and marked with a label to indicate its location. Specifically, ICL1 and ICL4 serve to connect the transmembrane helices in the N-terminal half of the protein to nucleotide-binding domain 1 (NBD1), while ICL2 and ICL3 link nucleotide-binding domain 2 (NBD2) to the transmembrane helices on the C-terminal (Loo & Clarke, 2014b). The specific helices connected to the ICLs and directly interacting with the NBDs are called intracellular helices (IHs). These IHs are labeled with numbers 1-4 to indicate their position and association with the respective ICLs. It is believed that the intracellular helices (IHs) and intracellular loops (ICLs) on ABC transporters function as "ball-and-socket" joints. Specifically, these structural elements facilitate the transmission of conformational changes occurring in the NBD to the TMD of the transporter (Loo & Clarke, 2014b). Loo et al. (2012) found that introducing single cysteine mutations into residues IH1 and IH3 and cross-linking them resulted in an active transporter (Loo et al., 2012). On the contrary, attempts at cross-linking IH2 with IH3 abolished the ATPase activity of the transporter (Loo & Clarke, 2014b). In addition, Pajeva et al. (2013) observed that the rotation of IH2 in *hPgp*

may be critical for binding ATP to the NBDs (Pajeva et al., 2013). This study also observed that conformational rearrangement and ATP binding led to the creation of a crucial interaction between Tyrosine 1087 (Tyr1087) in the NBD2 and Inner Helix 3 (IH3). This interaction was essential for the proper functioning of Pgp in transporting substrates out of the cell. For instance, mutating Tyr1087 to Phenylalanine (Phe) reduced Pgp-mediated ATPase activity, highlighting the critical role Tyr1087 plays in the ATP hydrolysis during substrate transport by Pgp (Loo & Clarke, 2014b, 2015; Pajeva et al., 2013).

Previous studies have also emphasized the importance of the transmembrane domains (TMDs) in facilitating the movement of substrates by the protein. Sajid et al. (200) proposed that transmembrane helices TMH1 and TMH7 are critical for the drug export function of this transporter. Mutating TMH1 and TMH7 resulted in Pgp variants that had a reduced ability to interact with a wide range of substances. For example, while some substrates like rhodamine could stimulate the protein, verapamil did not significantly affect the protein's basal activity (Sajid et al., 2018).

1.5 OVERCOMING P-GLYCOPROTEIN-MEDIATED MULTI-DRUG RESISTANCE

1.5.1 Physicochemical properties of P-glycoprotein substrates

Pgp can transport a wide range of structurally diverse substrates, making it challenging to predict and prevent drug interactions (Loo and Clarke, 2014). Understanding the properties of Pgp substrates is essential for predicting drug-drug interactions and designing strategies to overcome Pgp-mediated drug resistance in clinical settings. However, a few fundamental properties are shared among most Pgp substrates. Firstly, Pgp substrates tend to be lipophilic, meaning they have a high affinity for lipids and can easily permeate the cell membranes. Lipophilicity is critical in determining the interaction between P-gp and its substrates, as the protein's substrate-binding

pocket is primarily hydrophobic and can accommodate lipophilic molecules (Chufan et al., 2015; Sharom, 2014). Secondly, P-gp substrates can be positively or negatively charged depending on their chemical structure. However, charged substrates tend to have a lower affinity for P-gp than uncharged ones, as the protein's substrate-binding pocket is mainly hydrophobic and does not have a robust electrostatic component (Zhang et al., 2015). Also, Pgp can transport molecules up to a specific size, beyond which their transport is limited. The exact size limit varies depending on the protein's conformational state and the nature of the substrate (Zhang et al., 2015). Generally, Pgp can transport substrates with a molecular weight of up to 1000 Da. Pgp substrates can be structurally diverse, encompassing various chemical classes, including alkaloids, steroids, and anti-cancer drugs (Loo and Clarke, 2014). The protein can recognize and transport substrates with different chemical structures by combining hydrophobic and hydrogen-bonding interactions within its substrate-binding pocket (Sharom, 2014). Lipinski et al. (2001) identified five physiochemical properties shared by many successful drugs, including the Rule of 5, which predicts the pharmacokinetic properties of a drug (Leeson & Springthorpe, 2007; Lipinski, 2004). The Rule of 5 states that a drug candidate is more likely to be orally bioavailable if it has no more than one violation of the following criteria: (1) molecular weight less than 500 Daltons, (2) fewer than five hydrogen bond donors, (3) fewer than ten hydrogen bond acceptors, and (4) a calculated log P (octanol-water partition coefficient) less than or equal to 5. Pgp substrates encompass a diverse range of drugs, including but not limited to HIV protease inhibitors, opioids, antiepileptics, antibiotics, Ca²⁺ channel blockers, and anti-cancer therapeutics (Cirrito et al., 2005; D. C. Evans et al., 2003; Mahar Doan et al., 2002). Studies on the Structure Activity Relationship (SAR) of Pgp substrates show that these compounds are characterized by physicochemical properties that enable them to interact with Pgp and be transported out of cells (Eckford & Sharom, 2009; Seelig,

1998). Physicochemical properties shared by Pgp substrates include lipophilicity, aromatic rings, a high number of hydrogen bonding atoms, and a positively charged tertiary amine at physiological pH (Eckford & Sharom, 2009; Seelig, 1998). Not surprisingly, many drugs are substrates of P-glycoprotein (Pgp), considering the principles outlined in Lipinski's Rule of 5 applies to many successful drug candidates.

1.5.2 Available Strategies in Overcoming P-glycoprotein-Mediated Multi-drug Resistance

Developing effective strategies to overcome Pgp-mediated MDR is critical for improving the efficacy of chemotherapy and developing new therapeutic approaches.

1.5.2.1 Prodrug design

A prodrug is a pharmacologically inactive compound often designed to chemically improve the physicochemical or pharmacokinetic properties of the drug (Rautio et al., 2018). Once administered, the prodrug is metabolized by the body to produce the active form of the drug. The goal of prodrug design is to improve the therapeutic efficacy of drugs by enhancing physiochemical properties such as solubility, stability, absorption, and target-specificity of the drug. In addition, a prodrug can be designed to bypass Pgp-mediated transport by modifying drugs' binding affinity to Pgp or by being activated only after crossing the Pgp barrier (Husain et al., 2022; Q. Zhang et al., 2015). For instance, quinidine, a substrate of Pgp, showed about a three-fold uptake rate in MDCKII-MDRI cells compared to its prodrug, val-quinidine (Jain et al., 2004).

1.5.2.2 Global Inhibition of Pgp

Inhibition of Pgp has been proposed as a potential strategy that aims to increase the accumulation of drugs in cells by blocking the efflux activity of Pgp and overcoming MDR in cancer chemotherapy (Nanayakkara et al., 2018). The general strategy has been to develop

compounds that compete with anti-cancer drugs for transport or act as direct inhibitors of Pgp. Several Pgp inhibitors, such as verapamil, cyclosporine A, and tariquidar, have been identified and evaluated in preclinical and clinical studies (Lai et al., 2020). Inhibitors may competitively inhibit substrate extrusion by occupying protein transport binding sites, leaving no space for the P-gp and substrate interaction (Summers et al., 2004). Non-competitive inhibitors neither binds to the substrate binding sites nor are transported. They, instead, inhibit the protein efflux non-competitively by binding to an allosteric modulatory site (Bonito et al., 2023). Pgp may also be inhibited by disrupting ATP hydrolysis or modifying the cell membrane's lipid composition (Hoosain et al., 2015). However, the clinical application of Pgp inhibitors has been limited by their non-specificity, potential toxicity, and interactions with other drugs (Srivalli & Lakshmi, 2012). Often, Pgp inhibitors are co-administered with substrates to increase the bioavailability and efficacy of the therapeutic agents. Nonetheless, inhibitors can also lead to significant drug-drug interactions and toxicity (Liu et al., 2020).

1.5.2.3 Modification of the chemical structure of Pgp substrate

The activity of Pgp can reduce the effectiveness of drugs, especially in the treatment of cancer, as it decreases the concentration of the drugs within the cancer cells, making them less effective.

One way to address this issue is by altering the drug's physicochemical properties, such as its size, charge, and lipophilicity, to make it less likely to be recognized and transported by Pgp. For instance, reducing a drug's molecular size can help reduce its interaction with Pgp (Seelig, 2020). Pgp has a limited binding pocket that can only accommodate specific sizes of molecules. Hence, reducing the drug's size makes it less likely to fit into this pocket and be transported out of the cell. On the other hand, increasing the hydrophilicity of a drug molecule can make it less likely

to be transported across the cell membrane due to the relatively hydrophobic residues in the substrate binding sites of the transporter (Ahmed Juvalé et al., 2022). Thus, making the drugs more hydrophilic makes them less attractive to Pgp, resulting in their accumulation in the cell. For example, adding bulky hydrophobic groups to drugs can reduce their ability to interact with Pgp, thus increasing their intracellular concentration. For instance, daunorubicin and doxorubicin differ only by a single hydroxyl functional group, yet daunorubicin exhibits a transport rate that is 4 to 5 times higher than doxorubicin (Nguyen et al., 2020).

1.5.2.4 Targeted drug delivery

Tissue-targeted drug delivery can help circumvent the efflux function of P-glycoprotein, a membrane transporter protein that can pump drugs out of cells and reduce their effectiveness (Z. Zhao et al., 2020). Techniques aimed at delivering drugs directly to the tumor site or specific tissues make it possible to achieve higher drug concentrations locally and reduce the need for systemic administration, which can be limited by drug resistance and toxicity (Ulbrich et al., 2016). Additionally, targeted drug delivery can enhance the therapeutic index by reducing off-target effects and minimizing damage to healthy tissues (Nobili et al., 2012; Senapati et al., 2018).

There are several approaches to achieving tissue-specific drug delivery, such as the use of nanoparticles, liposomes, micelles, or polymer-based drug carriers, which can selectively accumulate in tumors or specific tissues through various mechanisms, including the enhanced permeability and retention effect (EPR), receptor-mediated endocytosis, or specific targeting ligands (Kapse-Mistry et al., 2014; Nobili et al., 2012; Senapati et al., 2018). These delivery systems can also provide sustained drug release and overcome the rapid clearance of drugs by efflux pumps. Tissue-specific drug delivery holds great promise for overcoming multi-drug resistance and improving the efficacy and safety of cancer treatment

1.5.2 Overcoming P-glycoprotein-Mediated Multidrug Resistance in Drug Design and Development: Clinical Implications

P-glycoprotein (Pgp) prevents many therapeutic drugs from reaching their targets, especially in cancer cells, which can block life-saving chemotherapeutic compounds from penetrating and accumulating to therapeutic levels necessary for the drug to be effective (Amin, 2013). Inhibiting Pgp may be a solution, but it can lead to drug accumulation and adverse side effects, making it impractical for clinical use (Y. Chen & Liu, 2012; Karthika & Sureshkumar, 2020; Kikuchi et al., 2013). Co-administering Pgp inhibitors with anti-cancer drugs have also been unsuccessful due to associated non-specificity and toxicities (Chung et al., 2016; Lai et al., 2020). Alternative methods to increase therapeutic drug permeability by evading Pgp-mediated transport exist but not without significant risks. Large or charged compounds can enter cells through adsorptive-mediated or receptor-mediated transcytosis, which is increased in the disease state, providing an advantage in treating disorders and diseases (Kapse-Mistry et al., 2014; Tachikawa et al., 2014; Waghray & Zhang, 2017). Nanoparticles targeting specific cell types can also be used, but this is a relatively new approach, with only a few nanoparticles currently in clinical trials for cancer treatment (Cavaco et al., 2017; Y. Chen & Liu, 2012). Evaluating the safety, risks, and benefits of these methods is necessary to improve patient treatment and quality of life. Understanding the structural basis of substrate recognition by Pgp is critical in designing compounds that circumvent Pgp-mediated transport or target-specific interfaces. This approach would avoid the pitfalls of previously established Pgp inhibitors and utilize small molecules instead of biotechnologies or biotherapeutics.

1.6 CONCLUSION

In conclusion, Pgp-mediated transport forms an important component of physiological mechanisms that protect sensitive tissues by limiting the entry of xenobiotics into systemic circulation. However, this mechanism also limits the permeability of many vital therapeutic agents, making Pgp a significant target in the pharmaceutical industry for overcoming multi-drug resistance and drug disposition. SAR studies have revealed the characteristics of Pgp substrates, which overlap with the properties of an ideal candidate for an efficacious drug (based on Lipinski's Rule of 5). This phenomenon explains why many drugs are Pgp substrates. Despite efforts to inhibit Pgp-mediated transport, global Pgp inhibition during clinical trials resulted in adverse side effects. It is, therefore, imperative to understand the protein-ligand interactions that drive Pgp-mediated transport. This knowledge is crucial to developing and administering next-generation drugs that can evade the protein and developing tissue-specific inhibitors with less toxicity. Furthermore, investigating the interaction between Pgp and its substrates, such as the camptothecins and vinca-alkaloids, will aid in developing better therapies that circumvent Pgp-mediated multi-drug resistance and drug disposition.

1.7 FIGURES

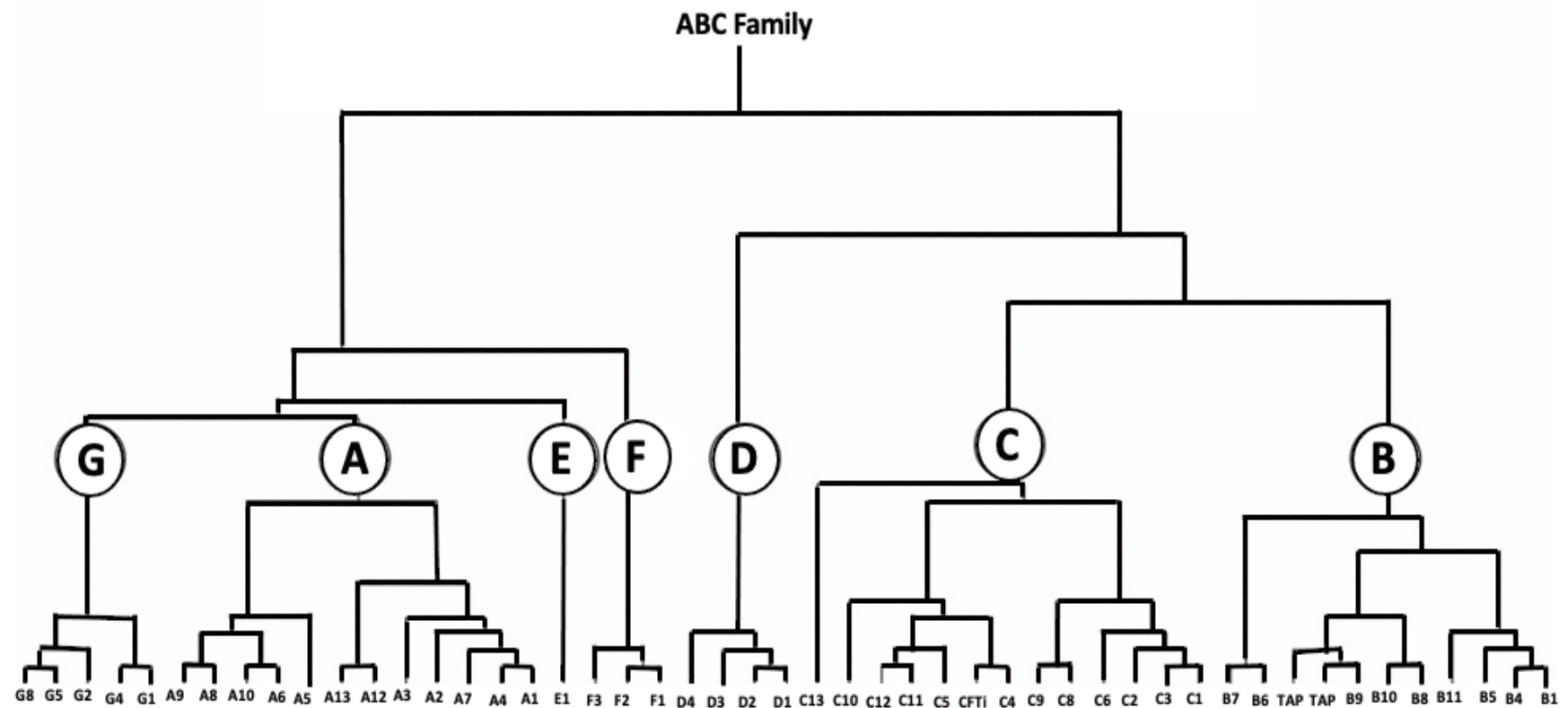


Figure 1.1 A dendrogram of cluster of existing human ATP-binding cassette (ABC) transporter genes

Figure 1.1 A dendrogram of cluster of existing human ATP-binding cassette (ABC) transporter genes. These genes have been categorized into families based on shared characteristics such as sequence similarity and the order of their discovery. The gene name 'B1' corresponds to 'ABCB1,' and so forth

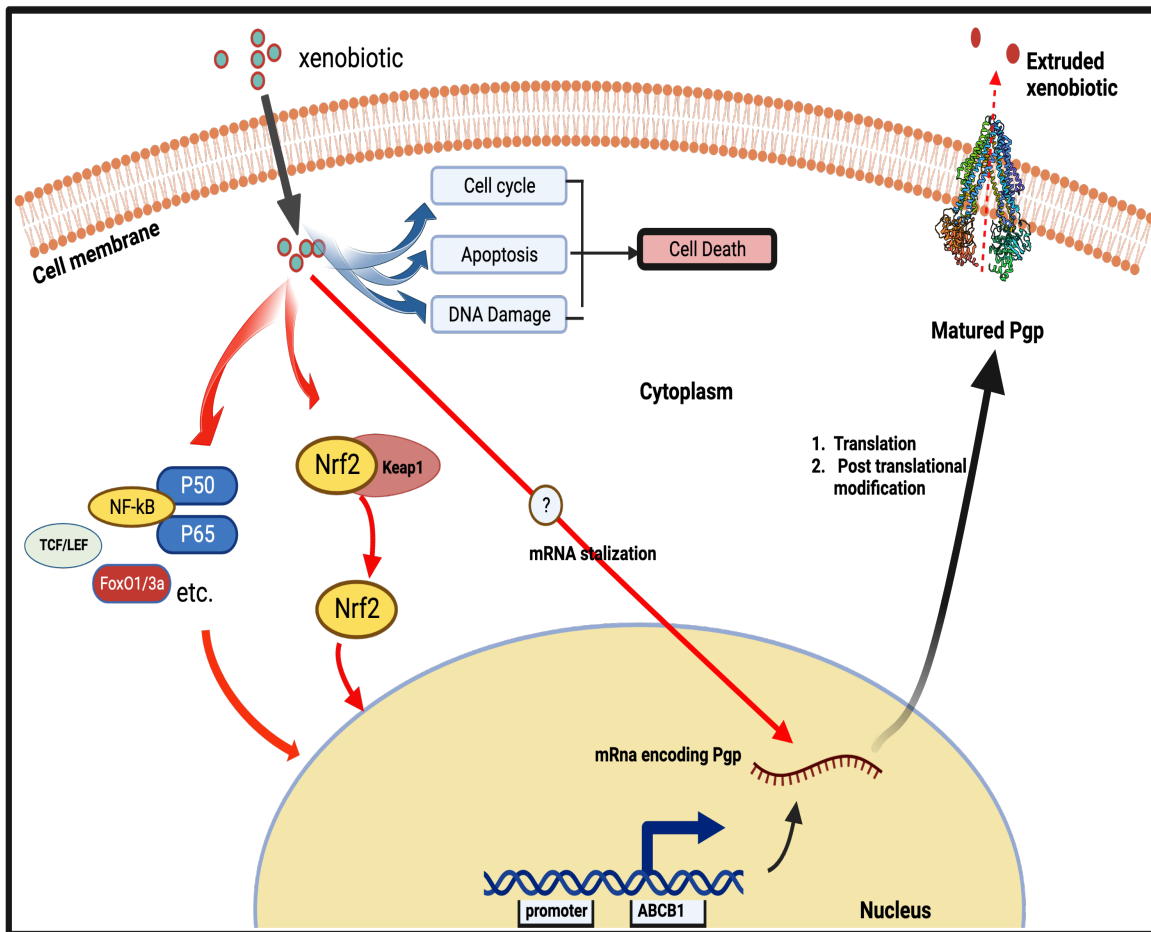


Figure 1.2 Effect of xenobiotics on different transcription factors that influence the expression of P-glycoprotein

Figure 1.2 Effect of xenobiotics on different transcription factors that influence the expression of P-glycoprotein. The "red arrows" indicate the mechanism through which xenobiotics enhance the expression and translation of Pgp. Conversely, the blue arrows depict the effect of anticancer drugs.

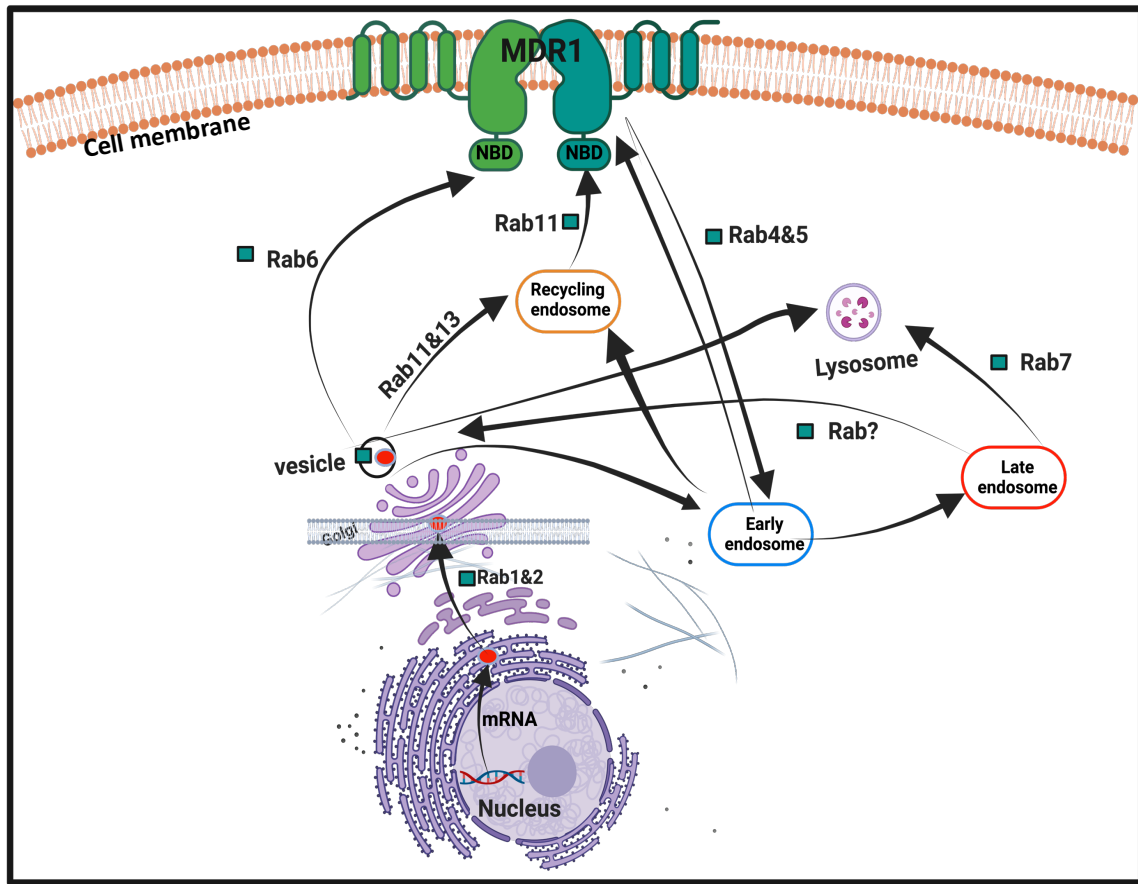


Figure 1.3 Illustration of the potential intracellular localization, trafficking, and recycling of P-glycoprotein

Figure 1.3 Illustration of the potential intracellular localization, trafficking, and recycling of P-glycoprotein (Pgp). Pgp is represented as a "red dot," and the various Rab GTPases assumed to play a role in Pgp trafficking and recycling are denoted as "teal squares." The black arrows depict the pathways of traffic and recycling within intracellular organelles and/or the plasma membrane. Shown in a green and teal in the plasma membrane Pgp embedded in the lipid bilayer

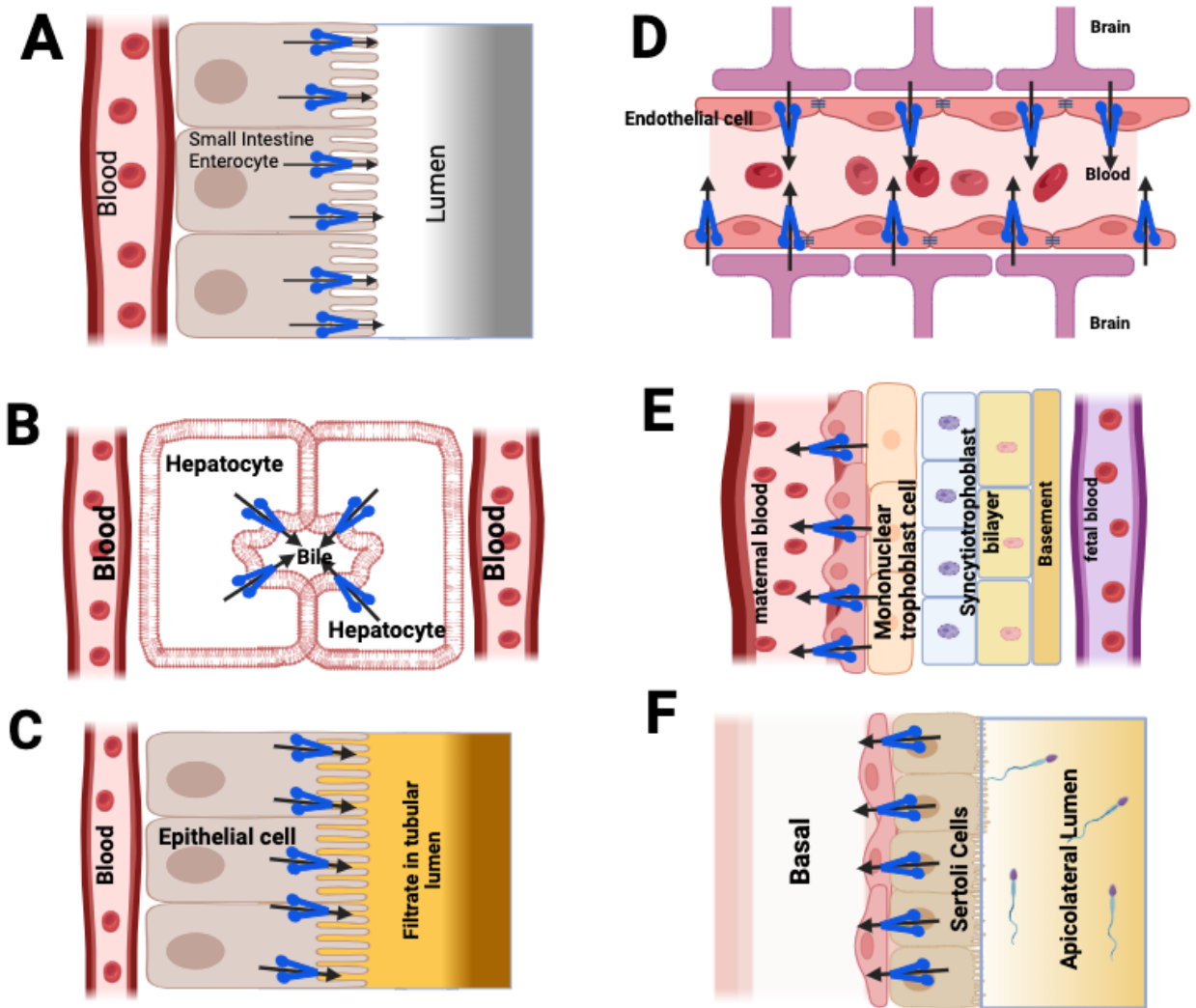


Figure 1.4 The distribution of P-glycoprotein in various tissues

Figure 1.4 The distribution of P-glycoprotein (Pgp) in various tissues. The distribution of Pgp at the (A) small intestine enterocyte, (B) bile canaliculus, (C) renal proximal tubule, (D) Blood Brain Barrier (BBB) endothelial cell, (E) Blood-Placenta Barrier (BPB) and (F) Blood-Testis Barrier (BTB) epithelial cell. Depicted in Blue, along with a black arrow, is Pgp indicating the direction substrate transport.

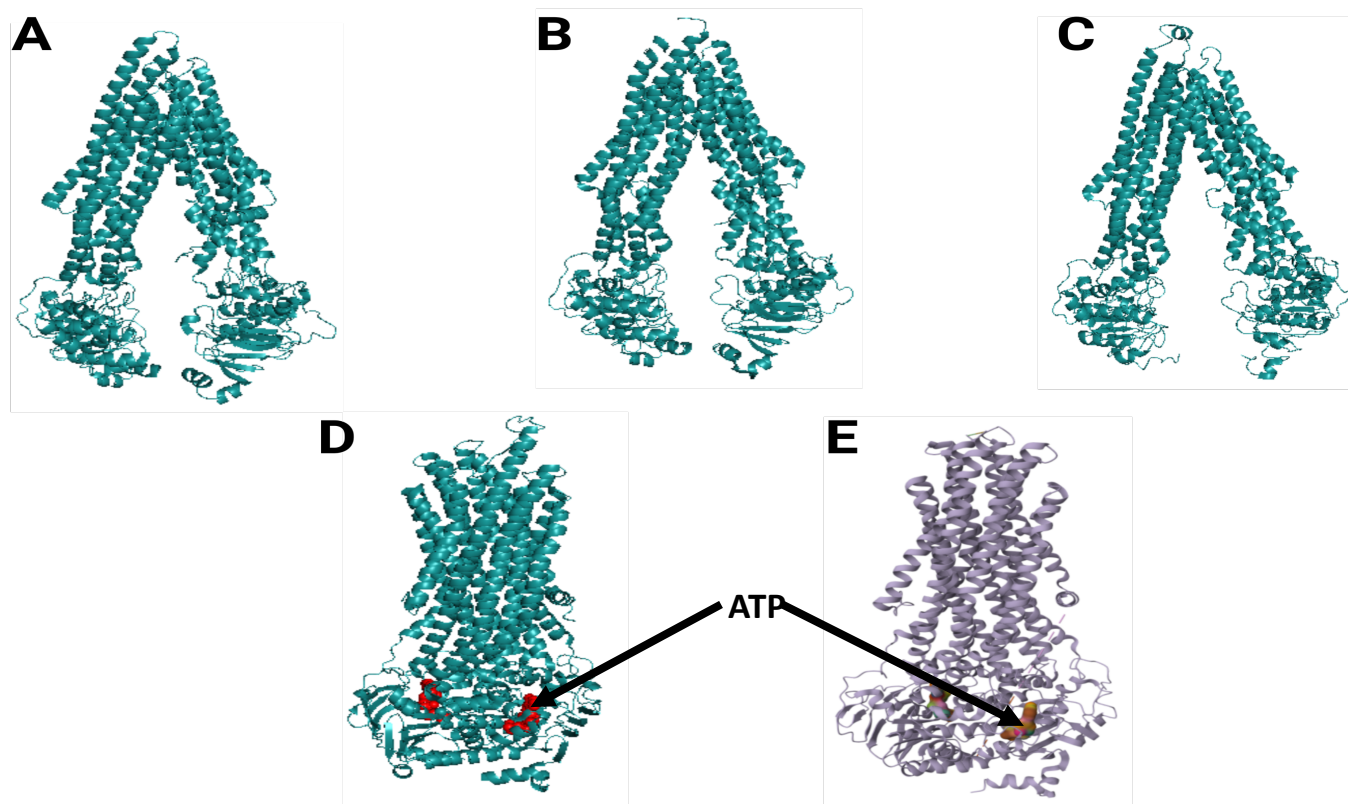


Figure 1.5 The open-inward and open-outward conformations of P-glycoprotein in Protein Data Bank

Figure 1.5 The open and closed conformations of P-glycoprotein (Pgp). A cartoon representation of the (A) inward-facing conformation of the first (PDB ID: 3G61) (B), refined (PDB ID: 4M1M), and (C) the most recent *m*Pgp (PDB ID: 7ZKB) deposited at the Protein Data Bank (<https://www.rcsb.org/>). The outward-facing conformation of (D) *m*Pgp (PDB ID: 7ZkB) and (E) *h*Pgp (PDB ID: 6C0V). *Mus musculus* and *Homo sapiens* Pgp cartoons are colored teal and heather, respectively. The ATP molecules bound into the NBDs are shown as Van der Waals spheres in (D) red or (E) brown

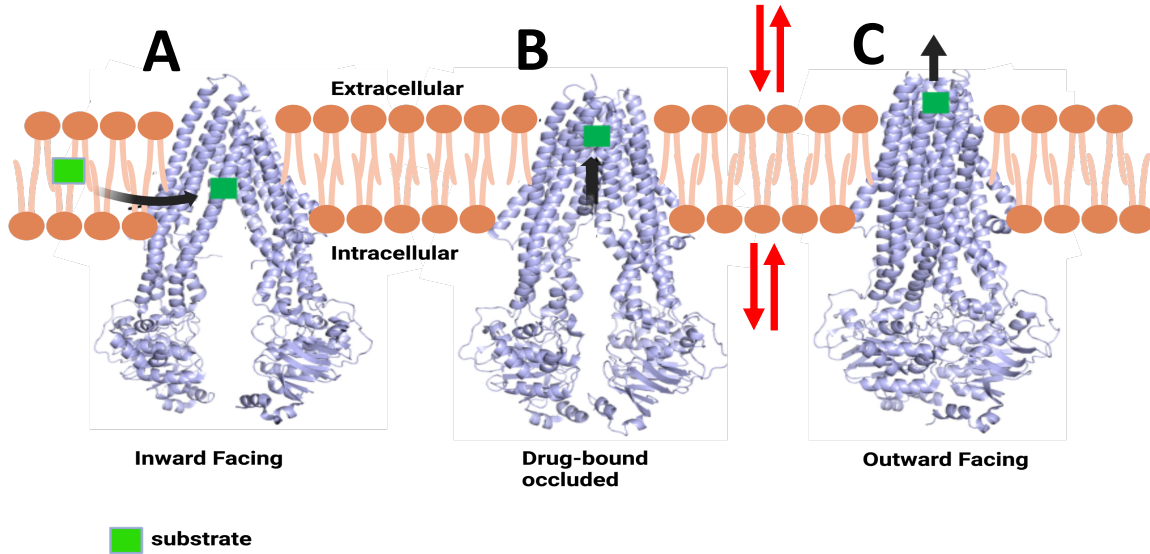


Figure 1.6 The substrate binding and conformational transport model of P-glycoprotein

Figure 1.6 The substrate binding and conformational transport model of P-glycoprotein (Pgp). Pgp in an (A) inward-facing orientation, (B) substrate-bound occluded conformation, and (C) ATP-bound outward-facing conformation. The red arrows show the passive diffusion of drug molecules, and the large black arrows reflect Pgp-mediated drug transport

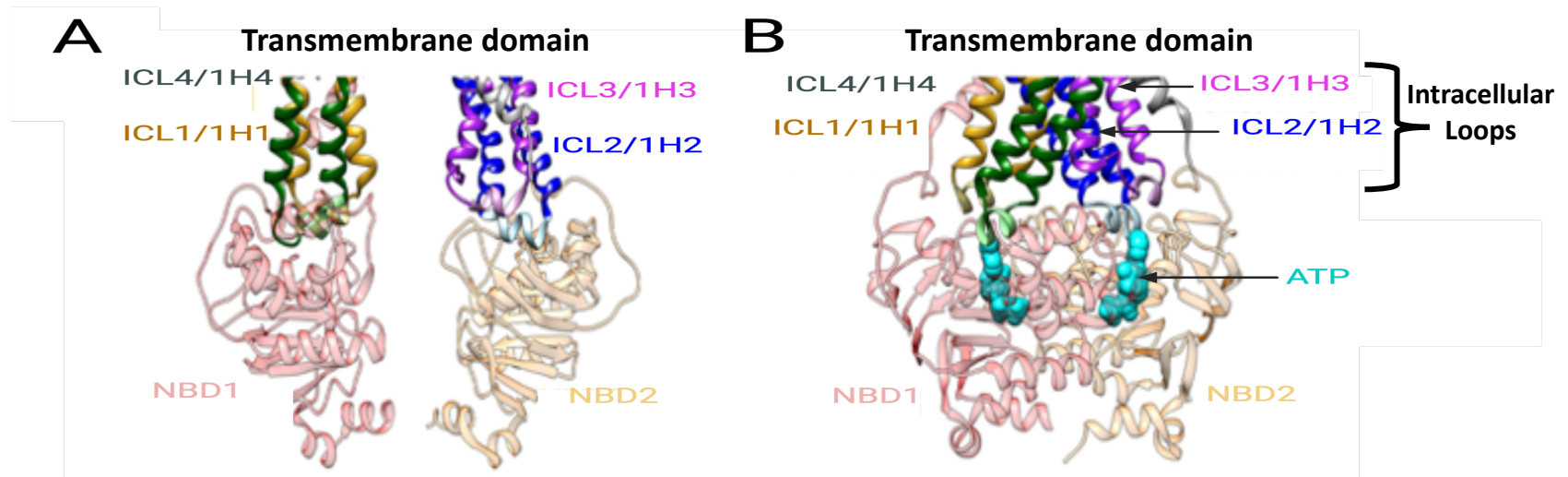


Figure 1.7 The crosstalk region between the TMD and the NBD of P-glycoprotein

Figure 1.7 The crosstalk region between the TMD and the NBD of P-glycoprotein (Pgp). The intracellular loops (ICLs) of Pgp in the (A) open (PDB ID: 4M1M) and (B) closed conformation (PDB ID: 6C0V). The ICLs are color coded by name. The light-colored regions on the ICLs are the intracellular helices (IHs) that directly interact with the NBD and/or ATP. The ATP molecules bound into the NBDs are shown as Van der Waals spheres in turquoise.

CHAPTER 2

DRUG-INDUCED CONFORMATIONAL DYNAMICS OF P-GLYCOPROTEIN UNDERLIES THE TRANSPORT OF CAMPTOTHECIN ANALOGS²

² Mensah, GAK., Schaefer, KG, Bartlett, MG, Roberts, AG, and King, GM (2023). Submitted to *International Journal of Molecular Sciences*, October 04, 2023

2.1 ABSTRACT

P-glycoprotein (Pgp) plays a pivotal role in drug bioavailability and multi-drug resistance development. Understanding the protein's activity and designing effective drugs require insight into the mechanisms underlying Pgp-mediated transport of xenobiotics. This study utilizes a conformationally-gated model to elucidate the Pgp-mediated transport of camptothecin analogs (CPTs). Pgp's conformational diversity is vast; herein, we use three model states: 'open-inward', 'open-outward', and 'intermediate'. Utilizing acrylamide quenching of Pgp fluorescence as a tool to examine the protein's tertiary structure, we observed that topotecan (TPT), SN-38, and irinotecan (IRT) induced distinct conformational shifts in the protein. TPT caused a substantial shift akin to AMPPNP, suggesting ATP-independent 'open-outward' conformation. IRT and SN-38 had relatively moderate effects on the conformation of Pgp. Experimental atomic force microscopy (AFM) imaging supports these findings. Further, the rate of ATPase hydrolysis correlated with ligand-induced Pgp conformational changes. We hypothesize that the distance separating the nucleotide-binding domain (NBDs) creates a conformational barrier for substrate transport. Substrates that reduce the conformational barrier, like TPT, are better transported. The affinity for ATP extracted from Pgp-mediated ATP hydrolysis kinetics curves for TPT was about 2-fold and 3-fold higher than SN-38 and IRT, respectively. On the contrary, the dissociation constants (K_{DS}) determined by fluorescence quenching for these drugs were not significantly different. STD NMR revealed comparable functional groups in TPT and IRT accountable for Pgp-CPTs interactions. Efforts aimed at modifying these functional groups, guided by available structure-activity relationship data for CPTs and DNA-Topoisomerase-I complexes, could pave way for the development of more potent next-generation CPTs.

2.2 INTRODUCTION

P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) superfamily that plays a crucial role in the development of multi-drug resistance (MDR) in chemotherapy (Benadiba & Maor, 2016). This transporter protein is believed to be present in more than 50% of tumor cells of patients undergoing chemotherapy (Bukowski et al., 2020). Studies have demonstrated a direct correlation between Pgp expression levels in cancer patients and adverse clinical outcomes in chemotherapy (Xia et al., 2019). Evolutionarily, Pgp plays a vital role in safeguarding cells against toxic insults (Abu-Qare et al., 2003; Georgantzopoulou et al., 2014). Thus, apical plasma membranes of cells that constitute essential physiological barriers and excretory tissues, including the blood-brain barrier, kidney, and liver, exhibit high expression levels of Pgp (Durmus et al., 2015; Fu, 2013). In a self-adaptive defense mechanism, tumor cells tend to increase the expression of Pgp to evade cell death (Chufan et al., 2015; Karthika & Sureshkumar, 2020; Robinson & Tiriveedhi, 2020). Regrettably, most chemotherapeutic agents are substrates of Pgp, a phenomenon that contributes significantly to the development of MDR phenotypic traits observed in cancer patients (Elmeliegy et al., 2020; Research, 2021). By actively pumping life-saving chemotherapeutic drugs out of cells, Pgp decreases the intracellular concentrations of drugs below the therapeutical levels required to be effective. Despite significant advancements in technology and research, the resistance of human malignancies to multiple chemotherapeutic agents remains a considerable barrier in cancer therapy (Bukowski et al., 2020; Mansoori et al., 2017) and potentially accounts for over 80% of treatment failures in patients on chemotherapy (Longley & Johnston, 2005).

Camptothecin (CPT) is a molecular scaffold for a class of anti-cancer drugs that effect its toxicity by targeting topoisomerase I (F. Li et al., 2017; Tesauro et al., 2019). During the S phase

of the cell cycle, camptothecin and its semi-synthetic derivatives (CPTs) selectively attach to and stabilize the topoisomerase-I-DNA complexes at the replication fork, resulting in potentially fatal breaks in DNA. (Laco, 2011; F. Li et al., 2017). In addition, new evidence suggests that CPTs may induce antitumor effects by alternative mechanisms, such as ubiquitin/26S proteasome-mediated degradation of topoisomerase-1 proteins (Ando et al., 2017). In general, the CPTs exist in two different pH-driven forms in solution - lactone and carboxylate (Kostjukov, 2021). The ability to passively diffuse into cells and exhibit antitumor properties is attributed to the lactone form (Kacprzak, 2013; Selvi et al., 2008).

Topotecan (TPT) and Irinotecan (IRT) are FDA-approved semi-synthetic CPTs frequently utilized as first- and second-line treatments for various types of cancer, including colorectal carcinoma (F. Li et al., 2017). TPT and IRT are more soluble, have improved bioavailability, and are less toxic compared to the parent compound (Kciuk et al., 2020; Parvez et al., 2021). IRT is a prodrug activated by the enzyme carboxylesterase to its active metabolite, SN-38, which is 100 to 1000 times more potent than the prodrug (Parvez et al., 2021). Given the crucial role of CPTs as effective anti-cancer drugs in chemotherapy, it's unsurprising that several other analogs, such as 9-amino camptothecin, are currently being developed and tested in various clinical trials (F. Li et al., 2017). Unfortunately, these analogs may suffer the same fate as the currently available antitumor agents if Pgp significantly transports them.

Although TPT, IRT, and SN-38 share the same basic structure, as illustrated in Figure 1, their pharmaceutical and pharmacological characteristics differ significantly (F. Li et al., 2017). Several membrane transporters can restrict the availability and distribution of drugs, including the CPTs (Mao et al., 2018). Pgp is a predominant efflux pump that eliminates CPTs from cancer cells (Kawahara et al., 2020; Lee et al., 2021). A retrospective clinical study on second-line

chemotherapy revealed that patients treated with IRT had a higher survival rate than those treated with TPT (Y. Zhao et al., 2019). Previous *ex vivo* studies have shown that IRT demonstrated greater efficacy than TPT in different human tumor xenograft models (Houghton et al., 1995). In addition, SN-38 has been reported to have > 2-fold higher cellular retention than TPT (Joto et al., 1997), while IRT showed about 2-fold tissue accumulation rate than SN-38 (Hirose et al., 2012). *In vitro* analysis using Pgp-transfected Caco-2 cells suggests that TPT has a Pgp-mediated efflux ratio about 6-fold higher than IRT (Lin et al., 2011). In LS513 cells treated with BCRP and MRP2 inhibitors, the relative efflux rate of TPT was approximately 4 times higher than SN-38 (Salphati et al., 2009). These results, taken together, suggest that the order of relative Pgp-mediated efflux rates for these CPTs is TPT > SN-38 > IRT. However, different cell lines and methods used in these transport measurements make detailed analysis and comparison difficult. Another significant drawback in these studies was the authors' inability to account for the mechanisms underlying the observed differences in reported efflux rates. So little is known about the CPTs that even the functional groups necessary for their interactions with Pgp are yet to be identified.

Our primary objective in this study was to identify the functional groups of CPTs that likely interact with Pgp. Identifying and modifying the functional groups could lead to the development of next-generation CPT analogs that have lower affinity to Pgp and are, therefore, better able to evade the transporter. Such analogs may have improved cellular retention and potency. Further, we investigated the molecular and structural mechanisms that drive Pgp-mediated transport of CPTs. Gaining a deeper understanding of the underlying mechanisms driving Pgp-mediated efflux is crucial for facilitating rational drug design aimed at circumventing Pgp-mediated MDR. To do this, we explored a potential drug-ATP cooperativity by measuring Pgp-mediated ATP hydrolysis in the presence of varying concentrations of TPT, IRT, and SN-38. To investigate structural

mechanisms underlying Pgp-mediated efflux of TPT, IRT, and SN-38, we examined the drug-induced conformational changes in Pgp. These conformational changes were studied using fluorescence spectroscopy and Atomic Force Microscopy (AFM). The binding affinity of CPTs to Pgp was estimated by probing the quenching of intrinsic Pgp fluorescence by the drugs. Finally, NMR saturation transfer double difference (STDD) was employed to identify functional groups on these drugs crucial in Pgp-drug interactions.

2.3 EXPERIMENTAL PROCEDURE

2.3.1 Chemical Reagents

Irinotecan hydrochloride salt trihydrate and SN-38 were obtained from LC Laboratories (Woburn, MA). Topotecan was obtained from Biotang Inc. (Albuquerque, NM). Adenosine 5'-(β,γ -imido)triphosphate lithium salt (AMPPNP), acrylamide, ammonium chloride (NH_4Cl), and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) were acquired from Sigma-Aldrich (Milwaukee, WI). Nitrilotriacetic acid (NTA) and diethylaminoethyl cellulose (DEAE) resin were purchased from Thermo Fisher Scientific. Ethylene glycol tetraacetic acid (EGTA) and imidazole were obtained from Alfa Aesar (Tewksbury, MA). Dithiothreitol (DTT) was purchased from Gold Biotechnology (Olivette, MO). Sodium orthovanadate (Na_3VO_4) was purchased from Enzo Life Sciences (Farmingdale, NY). The detergent, n-dodecyl- β -D-maltoside (DDM), was acquired from EMD Millipore Corporation (San Diego, CA). Disodium ATP, cholesterol, and Tris-HCl were purchased from Amresco (Solon, OH). Escherichia (E.) Coli total lipid extract powder was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Dimethyl sulfoxide-d₆ 99.9 atom % D and Deuterium oxide 99.9 atom % D were obtained from Sigma Aldrich, Burlington, Ma. All other reagents and chemicals not mentioned above but used in this study were purchased from Thermo-Fischer Scientific except otherwise stated.

2.3.2 Methods

2.3.2.1 Expression and Purification of P-glycoprotein

Strains of *Pichia pastoris* genetically engineered to overexpress histidine-tagged wild-type mouse Pgp (MDR3) were cultured in glycerol media and further induced with methanol to enhance the overexpression of the protein (Lerner-Marmarosh et al., 1999; Urbatsch et al., 1998) at the Bioexpression and Fermentation Facility (BFF), University of Georgia, Athens, GA. The cells were collected and lysed using a repeated cycle of freezing in liquid nitrogen, blending, and thawing (Dunn & Wobbe, 2001). The protein was then purified using a two-step approach previously described with modification (Bai et al., 2011; Lerner-Marmarosh et al., 1999). The first step involved an affinity chromatography technique with nickel-nitrilotriacetic acid (Ni-NTA) (ThermoFisher Scientific, Waltham, MA), followed by ion-exchange chromatography with diethylaminoethyl cellulose (DEAE) resin (ThermoFisher Scientific). Next, the purified proteins were solubilized in DDM and concentrated using Amicon Ultra-15 100 kDa cut-off filters EMD Millipore, Billerica, MA). The yield and concentration of the purified protein were determined using Nanodrop (DeNovix, PHL) and a molar absorption coefficient of 1.28 ml/mg (Bai et al., 2011). An SDS/PAGE analysis showed a purity of over 95% (Lerner-Marmarosh et al., 1999). The DDM-solubilized proteins were stored at -80°C.

2.3.2.2 Reconstitution of Pgp into Liposomes

Liposomes composed of 80% w/v Avanti E. coli total lipid extract and 20% w/v cholesterol, mixed in chloroform to a final concentration of 10mgml⁻¹ and a volume of 10mL, were made. The organic mixture was dried using a Buchi Rotavapor, Model R-114, and re-suspended with 10mL of rehydration buffer (0.1 mM EGTA and 50 mM Tris-HCl solution with a pH of 7.4).

The sample was freeze-thawed ten times using liquid nitrogen and a water bath to form liposomes of varying sizes, which were then extruded through a 400 nm cut-off filter at least 11 times using the LIPEX extruder (Northern Lipids, Burnaby, British Columbia, Canada) to produce homogeneously sized liposomes. Dynamic Light Scattering (DLS) was used to assess the sizes and uniformity of the extruded liposomes using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The DLS chromatograms were analyzed with Zetasizer 7.03 software and showed homogeneously sized liposomes with an average size of 250 nm.

Approximately 50 μ L of Pgp solubilized in detergent was dialyzed against HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 2 mM DTT, pH 7.4) for 2 hours to remove residual detergents. The dialyzed Pgp was mixed with 4 mgml⁻¹ of the previously extruded liposomes and incubated on ice for 1 hour to promote protein integration into the liposomes. Following incubation, the sample was dialyzed again on HEPES buffer for 2 hours to remove excess detergents and further enhance protein integration into the liposomes to form proteo-liposomes. The concentration of the proteo-liposomes was determined using a Bio-Rad DC Protein assay and aliquots were stored at -80°C in HEPES buffer, pH 7.4.

2.3.2.3 Pgp-Mediated ATPase Activity Assay

Pgp-mediated ATP hydrolyses in the presence of TPT, IRT, and SN-38 were measured using Chifflet's procedure as previously described (Chifflet et al., 1988) with some modifications. The Chifflet method estimates the activity of Pgp as a function of the concentration of inorganic phosphate, Pi, produced. Pi produced forms complexes with molybdenum, which characteristically has a high absorbance at 850nm. The absorbance was measured with a FlexStation (III) spectrometer (Molecular Device, Sunnyvale, CA) on a 96-well plate. 250 nM of reconstituted Pgp in Chifflet buffer (150 mM NH₄Cl, 5 mM MgSO₄, 0.02% wt/vol NaN₃, 50 mM Tris-HCl, pH 7.4)

were incubated at 37°C in the presence of varying concentrations of TPT, IRT, and SN-38, to determine the drug-induced ATPase activity of Pgp,. The final concentration of the reconstituted Pgp and ATP used were 50 nM and 3.2 mM, respectively. Control experiments containing vanadate and increasing concentrations of verapamil were run parallel to the samples. The ATPase activity kinetic curves were fit to a modified Michaelis-Menten equation ([Eqn1](#)) (Segel, 1993), and Igor 6.2 pro software (Wavemetrics, Tigard, Oklahoma, USA) was used for analysis.

$$V = \frac{V_{\max}[S]}{K_m + [S]} + V_{\text{basal}} \quad (1)$$

Where V is the ATP hydrolysis rate, $V_{\max}$ is the maximum ATP hydrolysis at saturating drug concentrations, [S] is the substrate concentration, K_m is the Michaelis-Menten constant, and V_{basal} is the basal ATPase activity.

2.3.2.4 Pgp-Drug Binding Affinity Determined by the Quenching of Protein Fluorescence

The physical properties of intrinsic tryptophan residues in proteins allow them to selectively absorb and emit around 295 nm and 330nm wavelengths, respectively. The binding of ligands to the protein can cause changes in the local environment experienced by the tryptophan residue(s) and subsequently affect the fluorescence intensity of the protein (Yammine et al., 2019). Intrinsic protein fluorescence quenching was used to determine the binding affinity of substrates/ligands, such as drugs and nucleotides, to Pgp as previously done (Gibbs et al., 2018; R. Liu et al., 2000; Sharom et al., 2003). Olis DM 45 spectrophotometer (Olis Corporation, Bogart, GA) was used to measure ligand-induced intrinsic tryptophan quenching of protein fluorescence. A 10 nm band-pass filter was placed on the excitation and emission paths of the spectrophotometer to mitigate the effect of Rayleigh bands on the observed fluorescence emission spectra. Sample solutions containing Pgp reconstituted into liposomes were diluted in Chifflet buffer supplemented

with 2 mM DTT to 1 μ M. Intrinsic protein fluorescence emissions were measured at 330 nm following excitation at 295 nm. Ligand-induced quenching of intrinsic protein fluorescence was corrected ($F_{corrected}$) for inherent factors such as background fluorescence, dilution, and inner filter effects using [eqn2](#) below (Lakowicz, 2011; Sharom et al., 2003):

$$F_{corrected} = (F - B)10^{\frac{(\epsilon_{ex}b_{ex} + \epsilon_{em}b_{em})[Q]}{2}} \quad (2)$$

Where F is the measured intrinsic protein fluorescence at 330 nm, $[Q]$ is the quenching ligand concentration, ϵ_{ex} is the molar absorption coefficient for excitation, and ϵ_{em} is the molar absorption coefficient for emission. The excitation (b_{ex}) and emission (b_{em}) path lengths were determined to be 0.4 mm and 1mm, respectively. Finally, B represents the background, the fluorescence obtained from the sample containing the solvent and the drug only. For TPT, the molar extinction coefficient at 295 nm and 330 nm were 6.074 $\text{mM}^{-1}\text{cm}^{-1}$ and 7.41 $\text{mM}^{-1}\text{cm}^{-1}$, respectively. The molar extinction coefficient of IRT at 295 nm and 330 nm were 6.02 mM^{-1} and 7.23 mM^{-1} , respectively. The estimated molar extinction coefficients of SN-38 were 3.86 mM^{-1} and 5.95 mM^{-1} at 295 nm and 330 nm, respectively. The extinction coefficient of AMPPNP at 295 nm was 0.03 $\text{mM}^{-1} \text{cm}^{-1}$ and nearly transparent beyond 300 nm.

Ligand-induced intrinsic protein fluorescence quenching often occurs via several mechanisms, the most common being static and dynamic quenching (Lakowicz, 2011). Static quenching occurs when the ligand/quencher forms a non-fluorescent complex with the fluorophore before excitation (Lakowicz, 2011). In dynamic quenching, ligand-induced fluorescence quenching is mediated by random collisions between the ligand and the fluorophore (Lakowicz, 2011). Several experimental methods could be used to determine the predominant mechanisms of quenching present. However, the effect of two or more different temperatures on the ligand-induced quenching of protein fluorescence was used in this study due to its effectiveness. The type

of quenching exhibited was mainly static for the three drugs used in this study (results not shown). The corrected fluorescence ($F_{corrected}$) quenching curves were fit into a modified Stern-Volmer equation below ([eqn3](#)).

$$F_{corrected} = \frac{F_{o(corrected)}}{1+K_{sv}[Q]} + F_{unquenched} \quad (3)$$

Where the $F_{o(corrected)}$ is the fluorescence in the absence of a quenching ligand, $F_{unquenched}$ is an offset related to unquenched fluorescence, $[Q]$ is the concentration of the ligand, and K_{sv} is the Stern-Volmer constant.

2.3.2.5 Drug-Induced Pgp Conformational Changes Probed Indirectly by Acrylamide Quenching of Protein Fluorescence.

The interaction between a ligand and protein can result in changes in the tertiary conformation of the protein and subsequently affect the availability of solvent-accessible intrinsic tryptophan residues within the protein to the bulk solvent. Acrylamide is a small polar collisional quencher that can be used to measure changes in the availability of solvent-accessible tryptophan residues within a protein (Cioni & Strambini, 1998; Strambini & Gonnelli, 2010). Since acrylamide cannot penetrate the hydrophobic interior of the protein (results not shown), changes in the fluorescence of tryptophan residues within the protein can help predict changes in the tertiary structure of proteins (Eckford & Sharom, 2008; Russell & Sharom, 2006). Acrylamide quenching of protein fluorescence was used to indirectly probe drug-induced Pgp conformational changes. For this assay, a solution containing 1 μ M of Pgp reconstituted into liposomes was titrated with acrylamide. The preparation was carried out in Chifflet buffer (pH 7.4) supplemented with 2 mM DTT. The assay was performed both in the presence and absence of ligand(s). Pgp fluorescence was monitored at 330 nm following excitation at 295 nm as the sample solution was titrated with increasing acrylamide concentration. The fluorescence intensities were corrected for dilution,

background, and inner filter effects using [Eqn3](#). A Stern-Volmer plot of $F_{o(corrected)}/F_{(corrected)}$ versus the concentration of acrylamide added was generated. The slope of the curve, an indicator of the degree of quenching, is related to the Stern-Volmer's constant (K_{sv}) by [Eqn4](#) (Lakowicz, 2011).

$$\frac{F_{o(corrected)}}{F_{(corrected)}} = 1 + K_{sv}[Q] \quad (4)$$

Where $[Q]$ is the concentration of the quencher, acrylamide.

2.3.2.6 Atomic Force Microscopy Imaging

Atomic force microscopy (AFM) has been adapted for analyzing protein dynamics and structure, which enabled the determination of conformational changes induced by ligands (Sigdel et al., 2018; Vahabi et al., 2013). In this study, Pgp reconstituted into liposomes was diluted in imaging buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂; pH 7.4) to 100 nM. About 90 μ L droplets of the diluted Pgp solution were deposited on a freshly cleaved mica surface (Grade v1; Ted Pella, Redding, CA), followed by 30 minutes of incubation at 25°C. This step caused the proteoliposomes to rupture, forming a planar lipid bilayer on top of the mica surface. The samples were rinsed at least 5 times using the buffer exchange technique, wherein 90 μ L of buffer was utilized. Next, AFM imaging of the EC-side and C-side domains of Pgp protrusions above the lipid bilayer was performed as previously described (Sigdel et al., 2018). For each of the selected CPTs used in this study, AFM imaging of the ligand-free Pgp (apoPgp) was performed first, then ligand was added, and imaging was repeated in nominally the same area. The amount of TPT, SN-38, and IRT added were 60 μ M, 30 μ M, or 100 μ M, respectively. Only AFM images that could be positively assigned as either EC or C-side domain features of Pgp were analyzed. All the AFM images were obtained in an imaging buffer at 25°C in tapping mode with biolever mini tips (BL-AC40TS, Olympus, Tokyo, Japan) installed on a commercial instrument (Asylum Research Cypher, Santa Barbara, CA). The tip-sample force was kept below 100 pN to minimize the

probability of protein distortion. The AFM images were analyzed with custom software written in Igor Pro 7 (Wavemetrics, Portland, OR), as described previously (Marsh et al., 2018; Sigdel et al., 2018). Kernel density estimation with Epanechnikov kernels in Igor Pro 7 software was used to generate smoothed histograms for further analysis (Chu et al., 2017). The histograms were fitted with a summation of the Gaussian distribution. The Bayesian information criterion was used to select the best model after fitting with 1-5 Gaussian distribution as described previously (Schaefer et al., 2023) (See Supporting Information).

2.3.2.7 Saturation Transfer Double Difference (STDD) NMR

STDD NMR is a robust ligand-focused technique used to empirically determine ligand-receptor interactions and epitope mappings (Roberts et al., 2011; Viegas et al., 2011). STD NMR involves the application of a long, low-powered pulse to selectively saturate the protons of the receptor protein (Roberts et al., 2011). For a molecule that binds to the receptor, only the protons within a distance of 5 Å from the saturated protein receive noticeable saturation transfer (Xu et al., 2021). The saturation transferred from the receptor to the bound ligands can be detected upon dissociation as saturation transfer difference (STD). The observed degree of saturation transferred inversely correlates with the distance between the ligand and the receptor and reflects the degree of interaction. Due to the high lipophilicity of most Pgp substrates, there is a high possibility of non-specific interactions between the ligands and liposomes used, which can interfere with the STD NMR spectrum observed. The STD NMR spectrum of the drug in the presence of liposomes was performed and subtracted from the STD ^1H NMR spectrum of the ligand in the presence of proteins reconstituted into liposomes to yield a saturation transfer double difference (STDD). This double subtraction accounts for the potential STD contributions from ligand-liposome interactions (Claasen et al., 2005; Haselhorst et al., 2007). This technique was used to probe the functional

groups of TPT and IRT involved in Pgp-drug interactions. All NMR experiments were carried out at 25°C (unless otherwise stated) on a Varian INOVA 600 MHz NMR spectrometer installed with ^1H -detect broadband probe {HX}, and the data were analyzed with [iNMR](#) software (Nucleomatic, Molfetta, Italy), Igor Pro 6.2, and [Mnova 14.2.0](#) (Mastrelab Research S.L, Santiago de Compostela, Spain).

The STDD NMR procedure in this study was performed as described by (Gibbs et al., 2018; Venkitakrishnan et al., 2012) with some modifications. The samples for STDD NMR experiments were prepared with 100 mM potassium phosphate buffer [80% D_2O , 20% ddH $_2\text{O}$, pH 7.4] containing 1 μM Pgp reconstituted into liposomes and 800 μM of either TPT or IRT. The control samples were identically prepared with liposomes instead of Pgp reconstituted in liposomes. A tailored gradient pulse sequence (WATERGATE) was included to suppress the background water signals (M. L. Liu et al., 1998; Piotto et al., 1992). The protein was selectively excited and saturated with a train of 50 ms gaussian-shaped-selective pulses for 2s, followed by a relaxation delay of 5s (Mayer & Meyer, 2001; Viegas et al., 2011). The STD NMR spectra were obtained via phase cycling by alternating the Pgp irradiation resonance between -1.5 ppm (on resonance) and 42 ppm (off-resonance) for a total of 512 scans (Gibbs et al., 2018). The difference between the on-resonance and the off-resonance created the STD ^1H NMR spectrum. A control experiment consisting of liposomes and drugs was obtained under identical conditions and subtracted from the ^1H STD NMR spectrum of the reconstituted Pgp sample with the drug to generate the STDD ^1H NMR spectrum. The difference observed (ΔI) directly correlates with the degree of interaction between the functional groups of drugs and the protein. The STDD amplification factor was calculated using the [Eqn5](#) below (Ledwitch, Barnes, et al., 2016; Mayer & Meyer, 2001).

$$STDD \text{ Amplification Factor} = \frac{[L] \Delta I}{[P] I_0} \quad (5)$$

Where I_0 is the amplitude of the ^1H NMR peaks, $[L]$ is the concentration of ligand, and $[P]$ is the protein concentration.

2.4 RESULTS

2.4.1. Effect of Topotecan, Irinotecan, and SN-38 on Pgp-coupled ATPase Activity

Pgp-mediated efflux is an ATP-dependent process that releases inorganic phosphates from ATP hydrolyses (Sauna & Ambudkar, 2007). Previous studies suggest that Pgp requires the hydrolysis of at least one molecule of ATP per complete catalytic cycle (Patzlaff et al., 2003; Sauna et al., 2002). Substrate-stimulated ATPase activity assays have been used previously to identify Pgp substrates/inhibitors (Litman et al., 1997; Seelig, 2020) and predict the rate of effective transport of substrates (Ambudkar et al., 1997). For most substrates, the rate of Pgp-coupled ATP hydrolysis correlates with transport (Kerr et al., 2001; Li-Blatter et al., 2012). An ATPase activity assay was used to determine the coupling effect of the rate of ATP hydrolysis on the Pgp-mediated transport of CPTs.

Figure 2 shows the effect of TPT, SN-38, and IRT on Pgp-mediated ATP hydrolysis. The kinetic curves generated from Pgp-mediated ATPase hydrolysis in the presence of TPT, IRT, and SN-38 were monophasic, suggesting a single drug binding site for these CPTs on the protein. In the absence of drugs (basal conditions), the average rates of Pgp-coupled ATP hydrolysis did were similar, averaging $555 \pm 9 \text{ nmolmin}^{-1}\text{mg}^{-1}$ (Fig. 2, Table 1). These basal rates of ATPase activities observed align well with the range observed in previous studies (Borgnia et al., 1996; Ritchie et al., 2009; Wilt et al., 2017). Kinetic parameters were determined by fitting Pgp-coupled ATP hydrolysis kinetic curves into a modified Michaelis-Menten equation ([Eqn1](#)) that accounts for the basal activity. For TPT (Fig. 2A), the estimated K_m from the kinetic curve was 21.51 ± 4.56

μM , closely resembling a previously reported value observed in CaCo-2 cells (H. Li et al., 2008). The V_{max} , representing the maximum rate of ATPase activity at saturating drug concentration, was estimated as $360 \pm 12 \text{ nmolmin}^{-1}\text{mg}^{-1}$ for TPT. As for SN-38 (Fig. 2B), the calculated K_m and V_{max} were $42.90 \pm 7.93 \mu\text{M}$ and $242 \pm 10 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively. In the case of IRT (Fig. 2C), the K_m and V_{max} were $58.52 \pm 14.81 \mu\text{M}$ and $167 \pm 12 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively. Although most Pgp substrates stimulate Pgp-mediated ATPase activity, a few have been reported to reduce it below basal levels (Anglicheau et al., 2006; Kerr et al., 2001; Sharom, 2011b). The ability to influence the rate of Pgp-mediated ATP hydrolysis in a dose-dependent, saturable manner, as seen with TPT, SN-38, and IRT, is a key characteristic shared by most Pgp substrates (H. Li et al., 2008; Srivalli & Lakshmi, 2012). These findings indicate that while CPTs reduce the ATPase activity rate below the basal level, the extent of reduction differs for each drug within the class. Overall, these results imply that the mechanisms underlying Pgp-mediated transport of CPTs also influence the rate of drug-induced Pgp-mediated ATP hydrolysis.

2.4.2. Determination of Drug-Pgp Binding Affinity by Protein Intrinsic Tryptophan Fluorescence Quenching.

The measurement of intrinsic protein fluorescence quenching by ligands is a robust spectrophotometric tool that has been used to determine protein-ligand binding affinities in previous studies (Gayer et al., 2020; Yammine et al., 2019). This spectrophotometric technique was used in this study to determine the apparent binding affinity of TPT, IRT, and SN-38 to Pgp. Figure 3 shows the analysis of Pgp fluorescence in the presence of increasing concentrations of TPT, SN-38, and IRT. Panels 3A, 3C, and 3E show the normalized Pgp fluorescence emission spectrum deduced in the presence of increasing concentrations of TPT, SN-38, and IRT, respectively. The protein fluorescence spectra showed a maximum fluorescence at 330 nm

following excitation at 295 nm, whereas the tested CPTs showed significant fluorescence beyond 380 nm in a dose dependent manner. In panels 3A, 3C, and 3E, the thick upper lines indicate the highest protein fluorescence levels observed in the absence of drugs. In the presence of 100 μM of TPT (Fig. 3A, lower thick lines), 60 μM of SN-38 (Fig. 3C, lower thick lines), and 100 μM IRT (Fig. 3E, lower thick lines), protein fluorescence decreased by approximately 71.6%, 40.6%, and 21.0%, respectively, from the initial fluorescence. The CPTs tested in this study were relatively insoluble in aqueous solution. The lower thick lines show the minimum protein fluorescence possible before any visible precipitation. In the presence of AMPPNP, 100 μM TPT, 60 μM SN-38, and 100 μM IRT decreased the protein fluorescence by 61.0%, 38.8%, and 21.7%, respectively (not shown). Figures 3B, 3D, and 3F show Pgp fluorescence monitored at 330 nm following excitation at 295 nm for increasing concentrations of TPT, SN-38, and IRT, respectively. Pgp fluorescence amplitudes at 330 were corrected for the inner filter effect using [Eqn2](#). The corrected fluorescence amplitudes ($F_{(corrected)}$) were plotted against the concentration of TPT (Fig. 3B), SN-38 (Fig. 3D), and IRT (Fig. 3F) in the absence (closed squared) and presence (opened squares) of saturating (3.2 mM) AMPPNP. The protein fluorescence in the presence of the CPTs analyzed in this study, either in the presence or absence of AMPPNP, showed a monophasic curve, which underpins our findings that CPTs likely have a single binding site on the Pgp. Subsequently, the protein fluorescence kinetic curves generated were fitted to [Eqn3](#) to estimate the Stern-Volmer's constant (K_{sv}). The maximum concentrations of the CPTs used for fitting were the highest permissible, given the notable inner filter effect exhibited by these drugs. The K_{sv} values from the Pgp fluorescence for TPT, SN-38, and IRT in the absence of AMPPNP were $0.41 \pm 0.09 \mu\text{M}^{-1}$, $0.28 \pm 0.09 \mu\text{M}^{-1}$, and $0.18 \pm 0.01 \mu\text{M}^{-1}$, respectively. In the presence of saturating AMPPNP, the estimated K_{sv} values decreased to $0.06 \pm 0.01 \mu\text{M}^{-1}$, $0.076 \pm 0.02 \mu\text{M}^{-1}$, and $0.07 \pm 0.01 \mu\text{M}^{-1}$ for

TPT, SN-38, and IRT, respectively. Since the K_{sv} values for the CPTs evaluated decreased significantly at a higher temperature (37°C) (results not shown), static rather than dynamic quenching was projected to be the dominant mechanism underlying the protein fluorescence quenching observed. For static quenching, the K_{sv} value correlates with the ligand-protein binding affinity (Campbell et al., 2012; Lakowicz, 2011). The estimated K_D for TPT (Fig. 3B) in the absence and presence of saturating AMPPNP were $2.47 \pm 0.57 \mu\text{M}^{-1}$ and $16.03 \pm 3.27 \mu\text{M}^{-1}$, respectively. The K_D recorded for SN-38 (Fig. 3D) was $3.53 \pm 1.14 \mu\text{M}^{-1}$, which increased to $13.20 \pm 4.23 \mu\text{M}^{-1}$ in the presence of saturating AMPPNP. For IRT (Fig. 3F), the K_D was determined to be $5.71 \pm 1.65 \mu\text{M}^{-1}$, which increased to $14.92 \pm 2.39 \mu\text{M}^{-1}$ in the presence of saturating AMPPNP.

2.4.3 Probing TPT, SN-38, and IRT-induced Pgp conformational changes using acrylamide quenching of Protein Fluorescence.

The tertiary structure of a protein may undergo conformational changes due to its interactions with ligands (Lakowicz, 2011; Sonveaux et al., 1999; A. Ward et al., 2007). Pgp-mediated translocation of substrates across the lipid bilayer has been reported to be modulated by ligand-induced conformational changes in the protein (Zoghbi et al., 2017). Acrylamide quenching of protein fluorescence is a spectrophotometric technique that has been used in previous studies to indirectly determine ligand-induced changes in the tertiary orientations of proteins (Akbar et al., 2016; Gibbs et al., 2018; Jazaj et al., 2019). The degree of acrylamide quenching is highest when the protein is in the open-inward conformation (R. Liu et al., 2000; Moller & Denicola, 2002) and least when the protein is in the open-outward conformation (R. Liu et al., 2000; Moller & Denicola, 2002; Wilt et al., 2017). Acrylamide quenching of protein fluorescence was used to test the hypothesis that Pgp-mediated translocation of TPT, SN-38, and IRT across the lipid bilayer is modulated by ligand-induced conformational changes in the protein.

Figure 4 shows the Stern-Volmer plots of drug-induced quenching of Pgp fluorescence by acrylamide in the presence of 60 μM TPT, 30 μM SN-38, and 100 μM IRT. The K_{sv} values were determined by fitting the Stern-Volmer kinetic curve into [eqn4](#) after accounting for the inner filter effect, dilution, and background with [eqn2](#). This value represents the slope and provides an estimation of the extent of quenching. Given that acrylamide is a small polar molecule with limited ability to penetrate the protein's hydrophobic core, a higher K_{sv} value indicates increased exposure of the tryptophan residues to the surrounding solvent. The Stern-Volmer curves shown in this study were linear, suggesting that acrylamide neither binds directly nor affects the conformation of the protein (Lakowicz, 2011). Acrylamide was titrated against the protein in the presence of tested CPTs and saturating (3.2mM) AMPPNP to assess the potential cooperative effects of AMPPNP on CPT-induced conformational changes in Pgp. The K_{sv} value obtained from the Stern-Volmer plot of NATA, a free analog of tryptophan, (S1A, dash-dotted line) represents a positive control and provides insight into the highest possible degree of quenching had all the tryptophan residues been fully exposed to the solvent. The estimated K_{sv} value for NATA was $15.43 \pm 0.37 \text{ M}^{-1}$, which aligns closely with findings from prior studies (Ledwitch, Barnes, et al., 2016; Patra et al., 2015; Strambini & Gonnelli, 2010). The estimated K_{sv} value for apoPgp (S1B, dash-line) and Pgp in the presence of saturating AMPPNP (S1B, dotted-line) were $1.58 \pm 0.03 \text{ M}^{-1}$ and $0.84 \pm 0.07 \text{ M}^{-1}$, respectively, consistent with previously published data (Russell & Sharom, 2006; Siarheyeva et al., 2010). Note that the significantly lower K_{sv} value observed for apoPgp in comparison to NATA indicates that only a fraction of the tryptophan residues within Pgp are accessible by the bulk solvent. The X-ray structures of mammalian Pgp and similar bacterial transporters devoid of ligands depict the transporter in a predominantly open-inward state, with notable distance between the two nucleotide-binding domains (NBDs) (Barbieri et al., 2021; Esser et al., 2017; J. Li et al.,

2014). We can infer from these findings that in the absence of ligands, Pgp predominantly adopts an open-inward conformation; thus, the K_{sv} value deduced for apoPgp represents the protein in an open-inward conformation. However, in the presence of saturating amounts of AMPPNP, there's a noticeable shift towards a primarily open-outward orientation. Since the analysis of both the x-ray structures (Moeller et al., 2015; Szewczyk et al., 2015) and the cryo-electron microscopy structure (Kim & Chen, 2018) of Pgp in the presence of AMPPNP consistently revealed a conformation characterized as open-outward, it is reasonable to infer that ApoPgp adopts an open-inward conformation.

The Stern-Volmer of apoPgp (Fig. 4, dashed-line) and Pgp in the presence of saturating AMPPNP (Fig. 4, dotted-line) are shown in each panel for comparison. Figures 4A (solid line) and 4B (solid line) show the Stern-Volmer plot of Pgp fluorescence induced by TPT in the absence and presence of saturating AMPPNP, respectively. Figures 4C (solid line) and 4D (solid line) show the effect of SN-38 on the Stern-Volmer plot of Pgp fluorescence in the absence and the presence of 3.2 mM AMPPNP, respectively. IRT-induced Stern-Volmer plot of Pgp deduced in the absence (Fig. 4E, solid line) and presence of 3.2 mM AMPPNP (Fig. 4F, solid line). In the absence of AMPPNP, the K_{sv} values for TPT, SN-38, and IRT were $0.78 \pm 0.06 \text{ M}^{-1}$, $1.12 \pm 0.02 \text{ M}^{-1}$, and $1.28 \pm 0.07 \text{ M}^{-1}$, respectively. The estimated K_{sv} values for TPT, SN38, and IRT in the presence of saturating AMPPNP were $0.71 \pm 0.04 \text{ M}^{-1}$, $0.81 \pm 0.08 \text{ M}^{-1}$, and $0.83 \pm 0.05 \text{ M}^{-1}$, respectively. Unlike SN-38 and IRT, the introduction of saturating concentration (3.2 mM) of AMPPNP did not have a notable effect on the K_{sv} value induced by TPT.

2.4.4 Ligand-Receptor Interactions Determined by STDD NMR

STDD NMR spectroscopy is a powerful technique frequently employed to determine epitope mapping and characterize ligand-receptor interactions (Mayer & Meyer, 2001; Monaco et

al., 2017; Xu et al., 2021). In the present study, STDD NMR was used to identify the precise molecular moieties on TPT and IRT that likely interact directly with Pgp. STDD NMR experiments for SN-38 were not feasible due to pronounced insolubility in most NMR-compatible solvents and instrumental limitations. Figure 5 shows the STDD NMR spectra and their corresponding amplification factors for TPT and IRT. For clarity, a labeled core structure of CPTs, accompanied by a corresponding legend highlighting the structural similarities shared by TPT, SN-38, and IRT, is shown in Figure 1. Panels 5A and 5B show the 1D ^1H STDD spectra of TPT and IRT, respectively. STDD spectra for TPT and IRT were compared with their respective 1D ^1H spectra (not shown) to identify the peaks that showed significant STDD. The 1D ^1H spectra observed for TPT and IRT were consistent with previously reported studies (D'Amelio et al., 2012; di Nunzio et al., 2013; Hyz et al., 2010). In the case of TPT, the saturation transfer was most pronounced for protons linked with C₁₅, C₁₈, C₂₄, C₂₅, C₂₇, C₃₀, and C₄₈ (see Fig. 1). Similarly, concerning the interaction between IRT and Pgp, substantial saturation transfer was observed for protons associated with C₁₅, C₁₈, C₂₃, C₂₄, C₂₇, C₃₄, C₃₇, C₄₅, and C₄₆ (Fig. 1). These findings imply that comparable functional groups on TPT and IRT likely play a pivotal role in their interactions with Pgp. The amplification factors for TPT and IRT were determined by fitting the ^1H 1D STDD spectral data to [Eqn5](#). Figures 5C and 5D show the STDD amplification factors for TPT and IRT, respectively. The highest amplification factors for TPT and IRT were approximately 133 and 81, respectively, while their average amplification factors were around 45 and 29, respectively. IRT had a higher number of protons with notable STDD signals compared to TPT, possibly attributable to the additional cyclic hydrophobic groups inherent in IRT. The intensity of STDD signals is inversely proportional to the sixth power of the distance between nuclei. Therefore, significant STDD signal intensity suggests shorter distances and tighter binding between the protein and

ligand. TPT-Pgp exhibited the highest STDD signal amplitudes, suggesting a higher binding affinity between TPT and Pgp. These results also support our Pgp- ligand affinity studies (Fig. 3) that showed that, among the three CPTs under study, TPT has the highest binding affinity to Pgp. Both TPT and IRT have a limited number of polar residues, implying that weak Van der Waals and hydrophobic interactions may play significant roles in their respective interactions with Pgp.

2.4.5 AFM analysis of the impact of Topotecan, SN-38, and Irinotecan on the conformational distribution of Pgp.

AFM is a high-resolution imaging technique used to study the topology of many structures ranging from single molecules to living cells (Dufrêne et al., 2017). This sensitive yet powerful multifunctional imaging platform has recently been used to study the conformations of proteins in the lipid layer (Pinho & Piedade, 2013; Sigdel et al., 2018). Consequently, AFM imaging was adopted in this study to explore the conformational changes Pgp undergoes as it extrudes ligands (TPT, SN-38, and IRT) across the lipid bilayer. AFM imaging was used to determine the drug-induced conformational shift in Pgp and ascertain the role these changes play in the transport of the CPTs. One limitation of X-ray crystallography is its reliance on the average coordinates of atoms to determine the crystal structures of biological molecules (Kondrashov et al., 2007). Unlike X-ray crystal structures, AFM imaging can sample through the various conformations of a biological molecule to identify changes in the external protrusion of proteins within a membrane (M. Li et al., 2017).

Figure 6 illustrates the conformational shifts in Pgp induced by CPTs. A representative AFM image of Pgp in the absence of a drug that highlights both the extracellular (EC; circled) and cytosolic (C; boxed) domains is shown in Figure 6A. Following previous work (Sigdel et al., 2018), the identification of the C-domains and EC-domains of the protein in the lipid bilayer was

achieved using protein height. C-side features were identified as those having heights between 55 and 90 Å. The approach has been verified using C-side-specific antibodies for Pgp (Sigdel et al., 2018). Smooth height histograms demonstrate height shifts in C-side features of Pgp in the absence and presence of TPT (Fig. 6B), SN-38 (Fig. 6C), and IRT (Fig. 6D). The population of the EC-domains of Pgp far outnumbered the C-domain, consistent with previously reported data (Sigdel et al., 2018). Conformational changes were more pronounced on the C-side. Therefore measurements taken from the protrusion on the EC-side were excluded from further analysis.

After the imaging of Pgp in the absence of ligands, one of the test compounds was added to the sample and imaged so that shifts may be observed in a nominally similar distribution of particles. This approach helps limit inherent differences observed in samples from batch to batch. The AFM imaging data collected and analyzed were fit in a model selection criterion (See Supporting Information, S2). Shifts in the tallest population are closely examined because the protein's height at the cytosolic region generally increases as the distance between the NBDs decreases, a key characteristic of Pgp in open-outward orientation. The changes in the height of Pgp at the C-side were tracked to identify the number of features occupying this state. Prior to the addition of TPT (Fig. 6B), the apo state ($N = 889$) had a population centered around 74 ± 9 Å. In the presence of 60 μ M TPT ($N = 780$), there was a change in height to 75 ± 7 Å, which is within error of the apo condition (Fig. 6E). However, there is a significant increase in the number of features sampled at the tallest heights. We indicate this using the change in the probability density (Δ PD; Fig. 6E) from apo to +TPT. Generally, the height of the protein at the cytosolic region increases as the distance between the NBDs decreases, a key characteristic of Pgp in an open-outward orientation. The increase in this population, with a Δ PD of 4.11×10^7 , implies that TPT shifts a larger fraction of C-side features toward an open-outward conformation as compared to

the apo condition. This process was repeated for the analysis of Pgp in the presence of SN-38 (Fig. 6C). Apo Pgp ($N = 217$) had Pgp with tall C-side height population centered around 84 ± 5 Å. The addition of 30 μ M SN-38 ($N = 229$) induced a decrease in the height of the taller Pgp features to 78 ± 7 Å. The fraction of features in this population decreased, with a Δ PD of -1.08×10^7 . Notably, the histograms show a distribution going from bimodal in the apo state to a broadened distribution in the presence of SN-38. The shift toward a more uniform histogram is, perhaps, indicative of a shift toward the intermediate conformation. Unlike the previous two drugs, IRT (Fig. 6D) shifts the height distribution of Pgp to the left of the height distribution histogram, where Pgp would be in a widely open-inward conformation. The tallest population with an average C-side height of 76 ± 9 Å in the apo state ($N = 237$) decreased to 70 ± 10 Å in the presence of 100 μ M IRT ($N = 320$). The Δ PD between these two populations is relatively low (4.91×10^6) when compared with similar conditions involving either TPT or SN-38, suggesting that the fraction of features that exist in this population, when compared to the apo condition, is more similar.

2.5 DISCUSSION

Pgp plays a crucial role in regulating the bioavailability of drugs and other xenobiotics, often leading to the development of MDR. To fully understand the activity of Pgp and encourage rational drug design aimed at circumventing the activity of the protein, several models that attempt to provide insight into the Pgp-mediated transport of xenobiotics have been proposed (Al-Shawi & Omote, 2005; Ritchie et al., 2011). Some of the widely used transport models include the partitioning-alternating model (Chufan et al., 2013; Safa, 2004; Verhalen et al., 2012) and ligand-ligand cooperativity (Shapiro & Ling, 1997). A conformationally-gated model was used in this study to provide a framework for the underlying mechanism driving Pgp-mediated transport of the CPTs. Mammalian Pgp, as well as analogous transporters in other organisms, can adopt a diverse

array of conformational states, both in the presence or absence of ligands (Frank et al., 2016; O'Mara & Mark, 2014). Thus, in a lipid environment conducive to the functionality of Pgp, a broad spectrum of diverse conformations would be present. However, for clarity and simplicity, one can model Pgp into three principal conformations: 'open-inward', 'intermediate', and 'open-outward'. The transporter protein is believed to be in an open-inward conformation when the nucleotide-binding domains are relatively apart, and the drug-binding pockets are exposed to the cytosolic region. An open-outward conformation of the protein is inferred when the nucleotide-binding domains are positioned closely together, and the ligand-binding cavities are accessible to the extracellular region of the cell. An intermediate conformation is established when the conformation of Pgp is between the open-inward and open-outward states, with the drug-binding region partially exposed to both the cytosolic and extracellular regions of the cell.

Acrylamide quenching of solvent-accessible tryptophan residues in Pgp reveals that TPT caused about 50 % decrease in the K_{sv} value of apoPgp (Fig. 5), akin to conformational change induced by AMPPNP. Since Pgp in the presence of AMPPNP (Gibbs et al., 2018; Sonveaux et al., 1999) is shown to be in a predominantly open-outward conformation, we propose that TPT induces a similar conformational shift in the protein. These findings are supported by the AFM data, which revealed an increase in the population of Pgp with the tallest heights ($> 73 \text{ \AA}$) when TPT was added (Fig. 6B). Logically, a reduction within the population with the shortest height ($< 70 \text{ \AA}$) was also observed (Fig. 6B). Of the three drugs tested, TPT showed the highest probability density difference (ΔPD) in the tallest populations (Fig. 6E, row 2), suggesting that the C-side features adopted more significant number of taller conformations. This finding implies that TPT induced open-outward conformation in Pgp. SN-38 and IRT caused $\sim 28\%$ and $\sim 20\%$ decrease in the K_{sv} value of the protein, suggesting that the active metabolite of IRT does not exert as much influence

on Pgp's conformation as TPT. Analyses of the AFM height distribution histogram reveal that whereas SN-38 slightly decreased both the tallest and shortest population of Pgp (Fig. 6C), IRT showed a relatively larger shift toward the left of the histogram (Fig. 6D). Further, SN-38 shifted the height distribution to a nearly unimodal histogram, inferring an intermediate conformational state. In contrast, IRT and its active metabolite induced minimal overall change in the ΔPD of the tallest population (Figure 6E, row 2).

A conformationally-gated model illustrating ligand-induced changes that drive Pgp-mediated transport of CPTs based on our findings is shown in Figure 7. Drawing from our observation and previously published studies (Futamata et al., 2020; Nguyen et al., 2020; Sigdel et al., 2018), we postulate that TPT induces Pgp conformational transition from a primarily open-inward conformation to an open-outward orientation. Conversely, SN-38 shifts the protein toward an intermediate conformation, between open-inward and open-outward conformation. IRT, on the other hand, moderately shifts Pgp to a conformational state closer to the open-inward orientation. These transitions are visually depicted in Figure 7D. Our structurally-gated model aligns well with the efflux ratios previously reported for TPT, SN-38, and IRT (Lin et al., 2011; Salphati et al., 2009). Furthermore, this model harmonizes well with the biochemical data presented in this study. For instance, previous studies established that Pgp-mediated ATPase activation may depend on the ligand-induced Pgp conformational changes (Pan & Aller, 2018; Zoghbi et al., 2017). The highest and lowest Pgp activation is likely when drugs induce open-outward and open-inward Pgp conformations, respectively. Thus, we theorize that the distance between the NBDs acts as a barrier that influences the transport of substrates. Substrates that reduce the conformational barrier, such as TPT, may be transported across the lipid bilayer at a faster rate, perhaps even without the binding of ATP. However, ATP binding and its subsequent hydrolysis would likely be required to

provide the energy required to reset the transporter for the catalytic cycle to resume. Interestingly, all the tested CPTs appear to slightly reduce the Pgp-coupled ATP hydrolysis below basal levels. Studies have revealed that the relatively high basal ATPase activity for Pgp could be attributed to the presence of endogenous substrates, such as cholesterol, in the lipid bilayer (Garrigues et al., 2002; Tran et al., 2023). These endogenous substrates are not transported by Pgp (Al-Shawi et al., 2003) but may be flip-flopped or shuttled between the two leaflets of the lipid bilayer by the protein (Sharom, 2014; Thangapandian et al., 2020). Thus, the detected ATPase activity of Pgp comprises both the basal activity prompted by endogenous substrates and the ATP hydrolysis induced by exogenous ligands. Following this logic, if a ligand interacts with the same sites as these endogenous substrates or binds to and restricts the flip-flop, it will seem to impede ATPase activity if its rate of ligand-induced ATP hydrolysis is slower than that demonstrated by the endogenous substrates. This theory is supported by earlier studies that suggest that the rate of Pgp-mediated ATPase activity is a function of a complex interplay between ligands, membrane lipids, and the protein (Li-Blatter et al., 2012) and the fact that Pgp exhibits high basal ATPase activity (Tran et al., 2023). We postulate that CPTs only appear to inhibit ATPase activity due to this phenomenon. Further research aimed at identifying the binding sites of both the ligands and endogenous substrates is needed to confirm this theory. The K_m for drug-induced Pgp-mediated ATPase kinetics curve (Fig. 2) revealed that TPT induced approximately 2- and 3-fold higher Pgp-ATP affinity than SN-38 and IRT, respectively. In terms of V_{max} , the order of Pgp-mediated ATP hydrolysis among the tested drugs was TPT > SN-38 > IRT. The analysis of Pgp-ligand binding affinity, as determined by the quenching of the protein's tryptophan residues, indicated that TPT exhibits the highest affinity for the protein, followed by SN-38. However, the differences in the

binding affinity of CPTs to Pgp observed were minor and are unlikely to be the primary driving mechanism underlying the differential transport rates reported for these drugs.

In the presence of 3.2 mM AMPPNP, the affinities of TPT, SN-38, and IRT for Pgp diminished by over three-fold, which suggests that the binding of AMPPNP non-competitively inhibits the binding of CPTs and, perhaps, enhances the release of the drugs from the protein-drug complex state to the extracellular environment. Previously reported data suggest that the binding of ATP analogs is sufficient to switch Pgp from high to low drug affinity conformation (Ahmed Juvalé et al., 2022; Bársony et al., 2016). Based on this analogy, it is likely that the binding of AMPPNP to Pgp either partially occludes or changes the local environment of the binding site of CPTs, thereby decreasing the protein-drug binding affinity. The changes in the drug binding sites may also be due to nucleotide-induced conformational changes in the entire Pgp structure (Futamata et al., 2020) rather than localized at the binding site. These hypotheses are supported by both the absence of substrate and the significant rearrangement of residues in the central binding cavity of Pgp observed in the solved open-outward crystal structure of Pgp (Kim & Chen, 2018). In the presence of saturating AMPPNP, both SN-38, and IRT induced significant changes in the protein conformation by shifting the protein into an open-outward state (Fig. 4). On the other hand, there was no notable change in the TPT-induced Pgp conformation in the presence of saturating AMPPNP. The lack of observable change in the presence of saturating AMPPNP may be due to the protein having already shifted to predominantly open-outward conformation in the presence of TPT. Thus, unlike SN-38 and IRT, which require the binding of ATP to shift Pgp into an open-outward conformation, TPT may be able to achieve this feat without ATP. This phenomenon may account for the differences in the transport rates observed for these drugs (Lin et al., 2011; Salphati et al., 2009). However, further ligand-induced conformational studies are

needed to confirm this theory. Remarkably, in the presence of saturating AMPPNP, all the tested CPTs induced similar open-outward conformation in Pgp (Fig. 4), highlighting the important role nucleotides play in the catalytic cycle of the protein.

The magnitudes for both the STDD amplitudes and the associated amplification factors estimated from the 1D ^1H STDD NMR analysis were higher for TPT than IRT, implying that TPT has relatively higher binding affinity to Pgp (Fig. 5). These observations align with the results from the binding affinity (Fig. 3), which indicate that TPT exhibits a slightly higher affinity to Pgp. Despite the E-ring of CPTs (depicted in Fig. 1) being widely considered the functional group responsible for the binding of CPTs to the DNA-topoisomerase-1 complex (Hecht, 2005), the groups responsible for drug-Pgp interactions remain elusive. The amplification factors derived from STDD NMR experiments are contingent upon NOE (Nuclear Overhauser Effect) transfer, a process that is inversely linked to distance. Among the various protons, those originating from C₁₅ (as depicted in Figs. 1 and 5) exhibited the highest interactions with Pgp. Altering these functional groups alone or along with others that displayed noticeable interactions with the transporter could lay the foundation for the development of the next generation of CPTs that can effectively evade Pgp-mediated efflux. While the prospect of developing enhanced drugs through this approach is promising, it comes with a limitation: the potential loss of drug functionality. Nonetheless, our understanding of the specific functional groups responsible for binding to Pgp, coupled with the existing structure-activity relationship studies involving CPTs and topoisomerase-DNA complexes, could lay the foundation for the development of potent next-generation CPTs capable of evading the transporter. To our knowledge, this study is the first to identify functional groups on TPT and IRT that likely interact with Pgp.

In summary, our results suggest that Pgp-mediated transport of CPTs is modulated by drug-induced Pgp conformational changes. TPT induces the highest conformational change in Pgp, reducing the NBD conformational barrier and subsequently stimulating ATP hydrolysis and transport. In comparison, SN-38 shifts the protein to an intermediate state, between open-inward and opened-outward. The intermediate conformation slightly reduces the conformational barrier, resulting in minimal translocation of SN-38 by the transporter. Conversely, IRT shifts the protein closer to the open-inward conformation and is poorly transported.

2.6 FIGURES

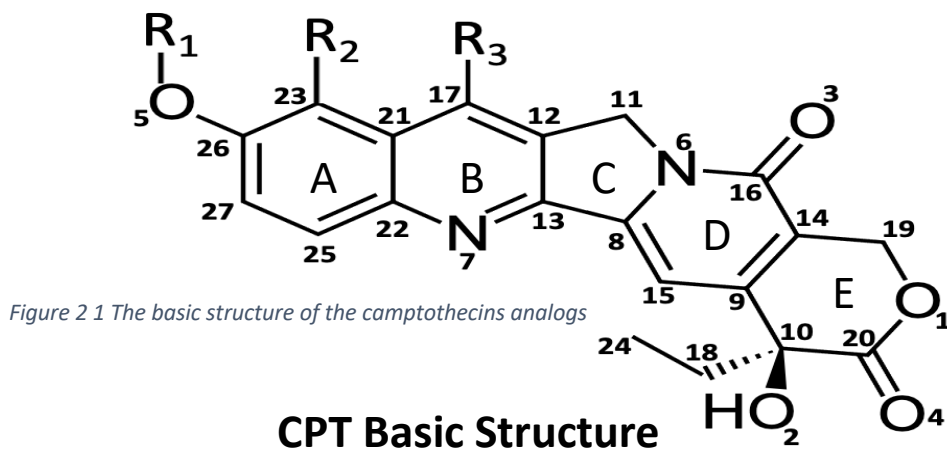


Figure 2.1 The basic structure of the camptothecins analogs

Camptothecin Derivatives	Substituents		
	R ₁	R ₂	R ₃
TPT	²⁸ H-	³⁰ ²⁹ (CH ₃) ₂ N-	⁴⁸ H-
SN-38	²⁸ H-	⁴⁵ H-	⁴⁶ ⁴⁷ CH ₃ CH ₂ -
IRT		⁴⁵ H-	⁴⁶ ⁴⁷ CH ₃ CH ₂ -

Figure 2.1. The basic structure of the camptothecins analogs. The table shows distinctions between TPT, SN-38 and its pro-drug, IRT.

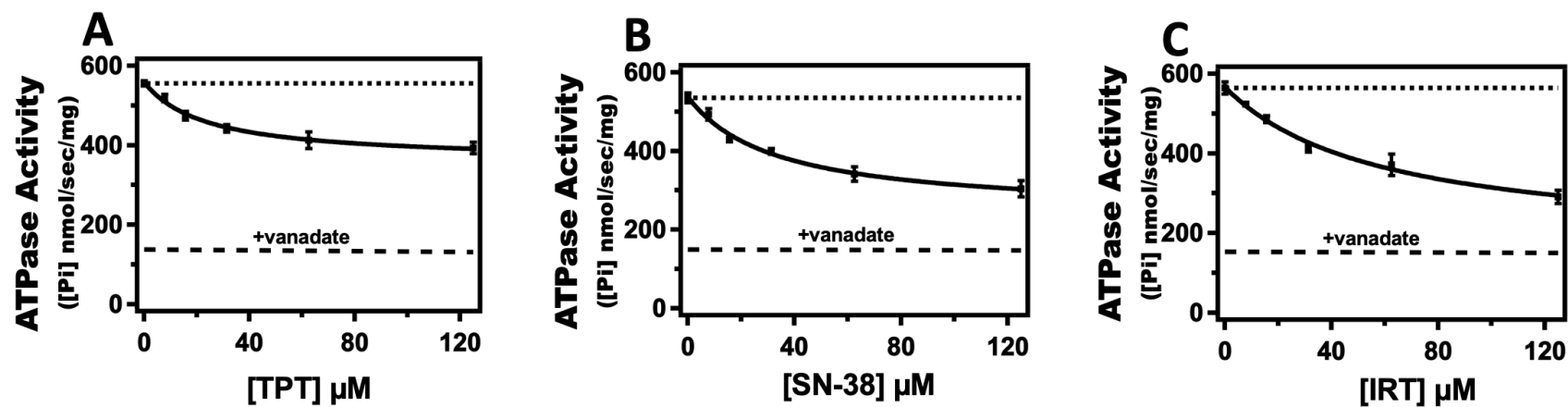


Figure 2.2 The effect of TPT, SN-38, and IRT on Pgp-mediated ATP hydrolysis

Figure 2.2. The effect of TPT, SN-38, and IRT on Pgp-mediated ATP hydrolysis. Ligand-induced Pgp-coupled ATPase activity in the presence of varying concentrations of (A) TPT (closed squares), (B) SN-38 (closed squares), and (C) IRT (closed squares). For comparison, each panel shows the basal activity level recorded in the absence of ligands as a dotted line. The dashed line in each panel represents the average ATPase activity in the presence of 200 μM of vanadate. Each data point represents the average of at least 3 independent experiments, and the error bars shown represent the standard deviation.

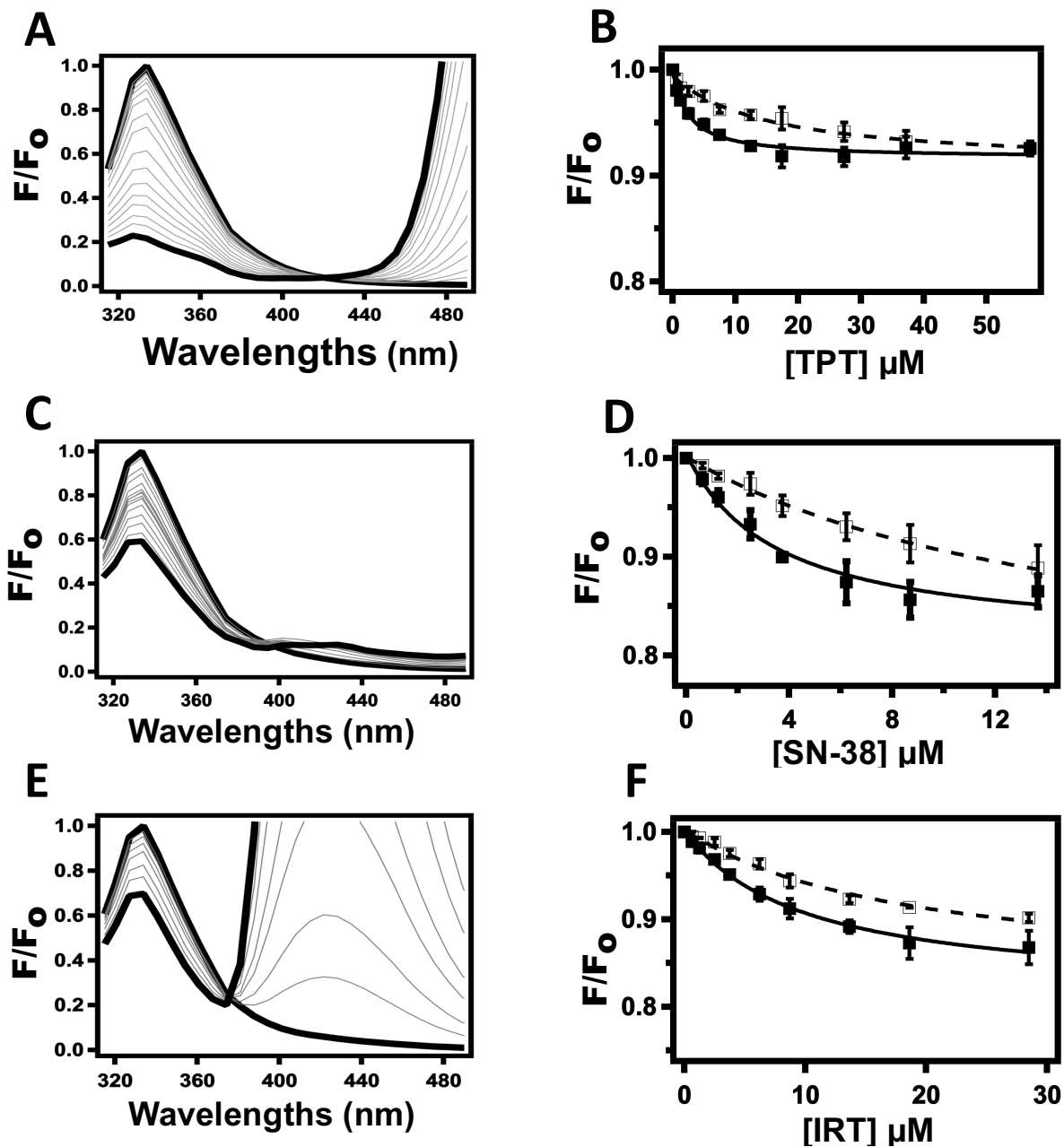


Figure 2.3 The binding affinity of TPT, SN-38, and IRT to Pgp determined by the quenching of intrinsic protein fluorescence

Figure 2.3. The binding affinity of TPT, SN-38, and IRT to Pgp determined by the quenching of intrinsic protein fluorescence. Fluorescence emission decay spectra of Pgp following excitation at 295 nm in the presence of an increasing concentration of (A) TPT, (C) SN-38, and (E) IRT. The upper and lower thick lines represent the absence (0 μM) and the presence of saturating TPT concentrations, respectively. Fluorescence spectra at intermediate concentrations are shown in

gray lines. The corrected fluorescence emission amplitudes at 330 nm were plotted as a function of the concentration of (B) TPT, (D) SN-38, and (F) IRT in the absence (closed squares) and presence (open squares) of 3.2 mM AMPPNP. Each data point and error bar represent the average and standard deviation of at least three independent experiments.

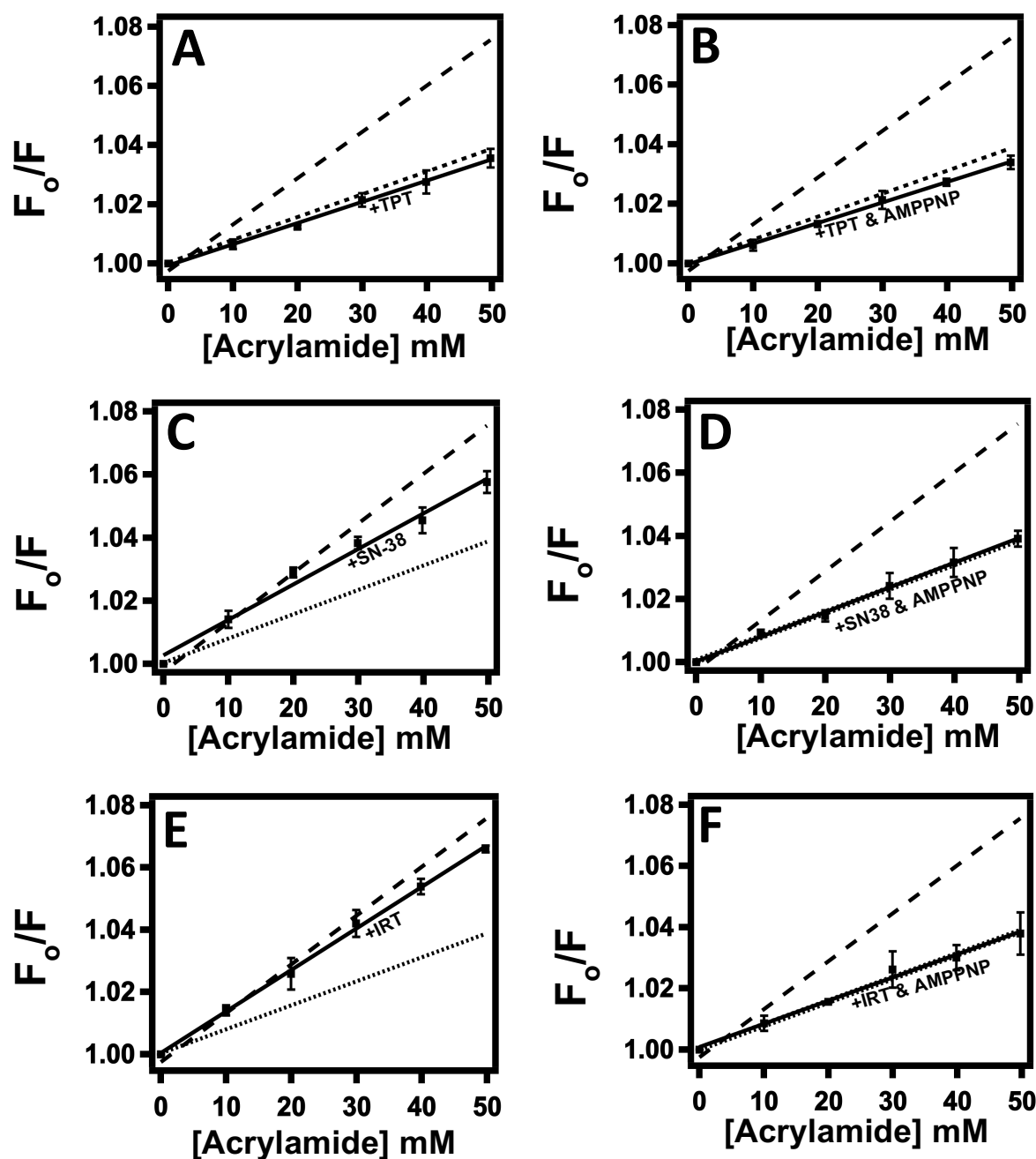


Figure 2.4 Camptothecin-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence

Figure 2.4. Camptothecin-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence. Stern-Volmer plots of Pgp induced by TPT are shown in the (A) absence and (B) presence of saturating AMPPNP. (C) and (D) show SN-38-induced Stern-Volmer plots of Pgp in the presence and absence of AMPPNP, respectively. The Stern-Volmer

plots of Pgp induced by IRT are shown in (E) absence and (F) presence of AMPPNP. For reference, the Stern-Volmer plot of apoPgp in the absence (dashed line) and the presence of AMPPNP (dotted line) are shown in each panel. The TPT, SN-38, IRT, and AMPPNP concentrations used were 60 μ M, 30 μ M 100 μ M, and 3.2 mM, respectively. The data points and error bars represent the mean and standard deviation for at least three independent experiments.

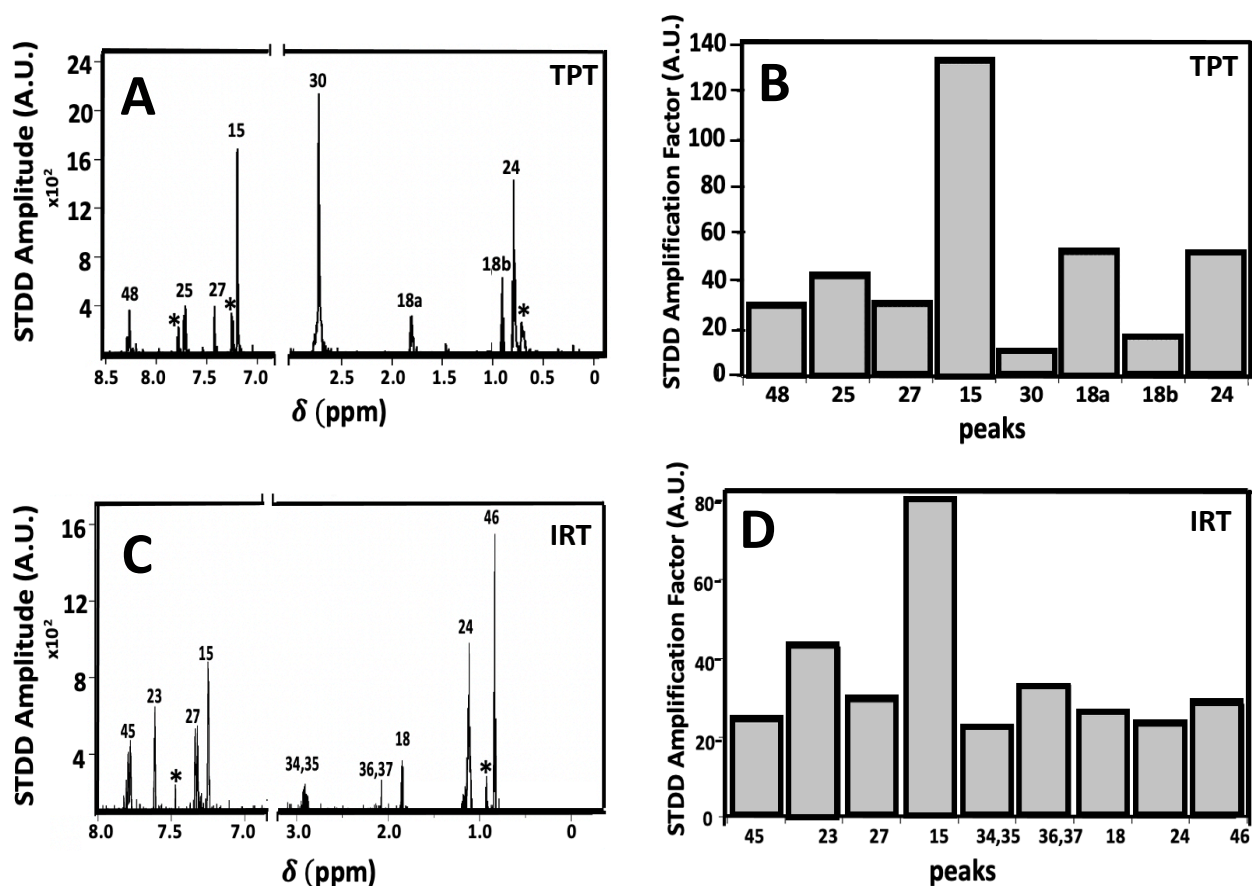


Figure 2.5 The interactions of camptothecin analogs with Pgp were investigated using STDD NMR spectroscopy

Figure 2.5. The interactions of camptothecin analogs with Pgp were investigated using STDD NMR spectroscopy. The 1D ^1H STDD NMR spectra for TPT and IRT are shown in (A) and (C), respectively. Amplification factors calculated from the STDD data are shown for (B) TPT and (D) IRT. The labels on the peaks in each panel correspond to the proton labels in Figure 1. The sub-labels 'a' and 'b' indicate axial and equatorial protons, respectively. The '*' denotes unidentified peaks.

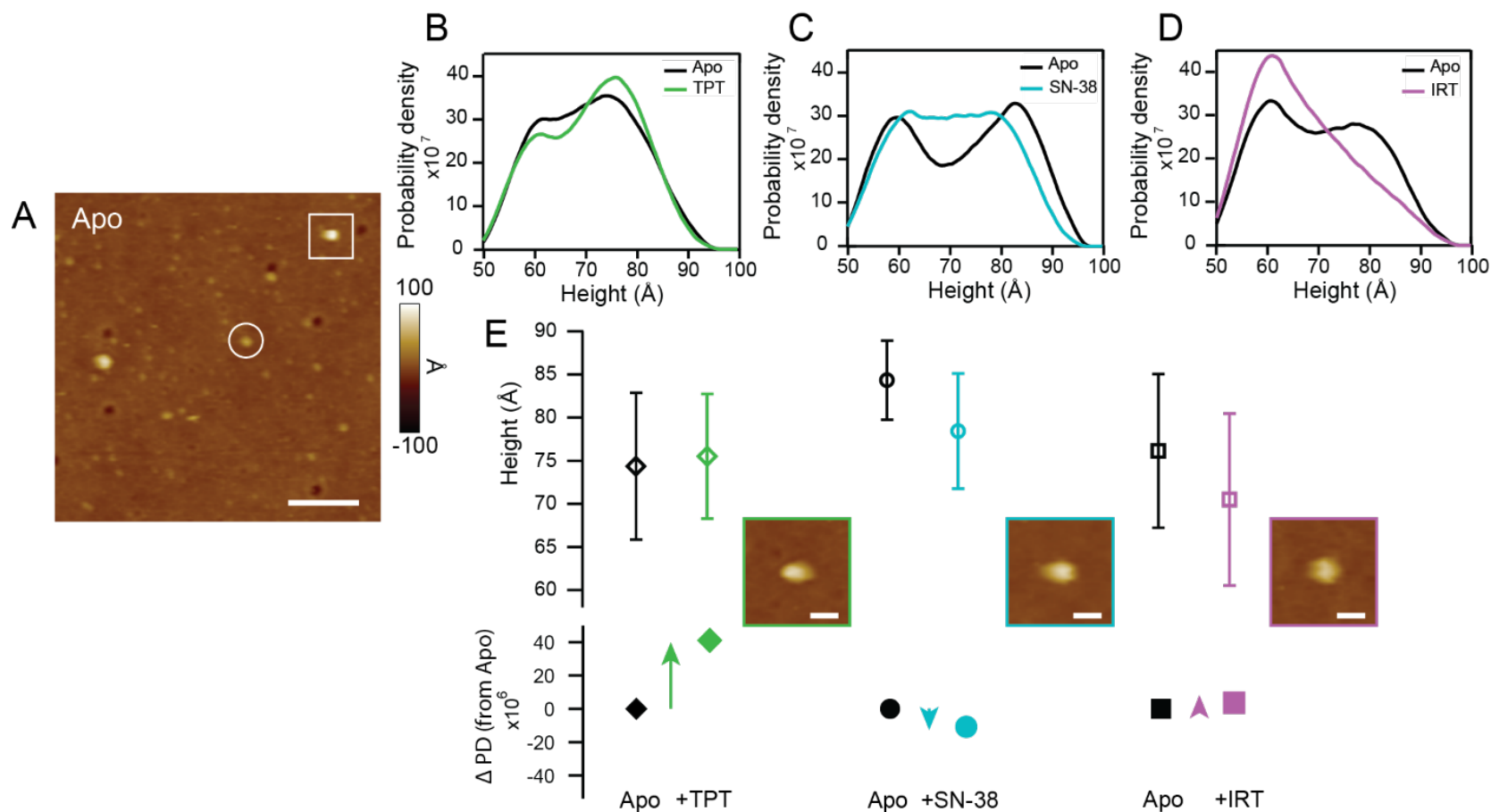


Figure 2.6 The effect of TPT, SN-38, and IRT on the height distributions of Pgp for the C-domain protrusions determined via AFM

Figure 2.6. The effect of TPT, SN-38, and IRT on the height distributions of Pgp for the C-domain protrusions determined via AFM.

(A) An AFM image of apo Pgp indicates representative C-side (boxed) and EC-side (circled) features; scale bar = 100 nm. Smooth histograms show population shifts from apo (black) in the presence of TPT (B; green), SN-38 (C; blue), and IRT (D; purple). Histograms

are integrated to unity area using SI units such that the y-axis is the probability density. (E) The heights of the tallest populations are compared side-by-side to show relative shifts (top row; open shapes); error bars are the standard deviation of the individual Gaussian distribution for that population. Insets show representative C-side features within the tallest population after the addition of each CPT; scale bars = 20 nm. The probability density difference (ΔPD) relative to the apo state indicates how the fraction of features within these tall populations shifts in the presence of each CPT (bottom row; closed shapes).

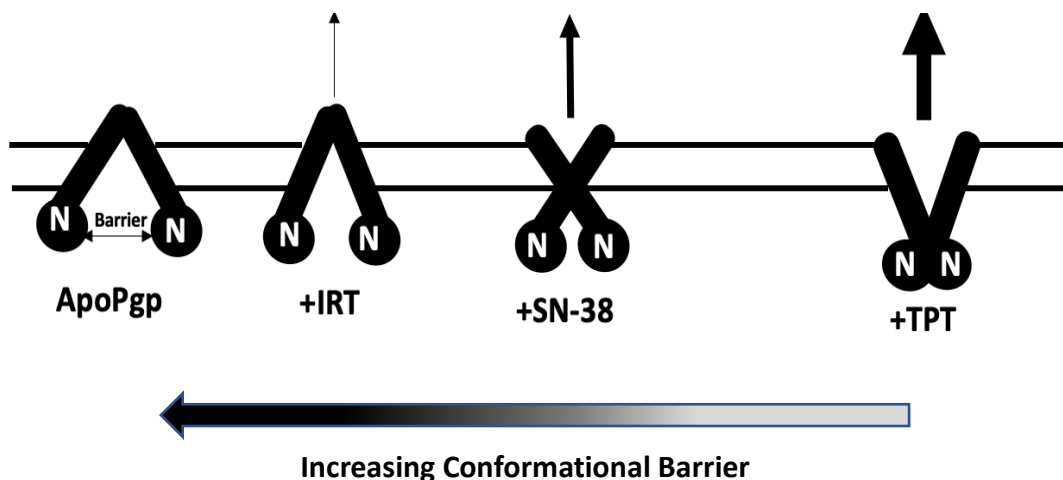


Figure 2.7 A cartoon depiction of a conformationally-gated model of Pgp conformations in the lipid bilayer in the presence of TPT, SN-38, and IRT

Figure 2.7. A cartoon depiction of a conformationally-gated model of Pgp conformations in the lipid bilayer in the presence of TPT, SN-38, and IRT. Pgp is represented in solid black, with the black circular region labeled 'N' representing the nucleotide-binding domain (NBD). The horizontal arrow indicates the conformational barrier. The two horizontal lines represent the lipid bilayer. The black arrows and its thickness reflect the direction and rate of transport, respectively.

Table 2.1. Kinetic parameters and the corresponding averages deduced by fitting the rate of Pgp-coupled ATPase activity over a range of CPTs concentrations (Fig. 2).

CPT Analogs	Basal ATPase Rate (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
TPT	559 ± 6	21.51 ± 4.56	360 ± 12
SN-38	538 ± 10	42.90 ± 7.93	242 ± 10
IRT	567 ± 10	58.52 ± 14.81	167 ± 12

2.7 SUPPLEMENTARY INFORMATION

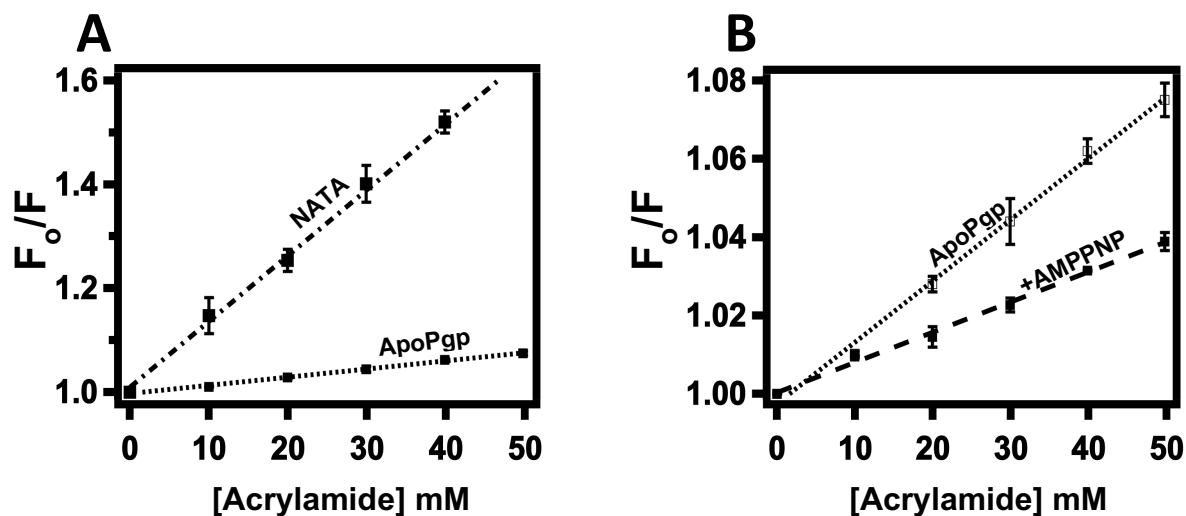


Figure S2.1 Pgp conformational changes probed by acrylamide quenching of tryptophan residue fluorescence

Supplemental Figure S2.1 Pgp conformational changes probed by acrylamide quenching of tryptophan residue fluorescence. (S1A) Stern-Volmer plot of NATA (dash-dotted line), and ApoPgp (dashes). (S1B) Stern-Volmer Plot of ApoPgp (dashes) and Pgp in the presence of saturating (3.2 mM) AMPPNP (short-dashes).

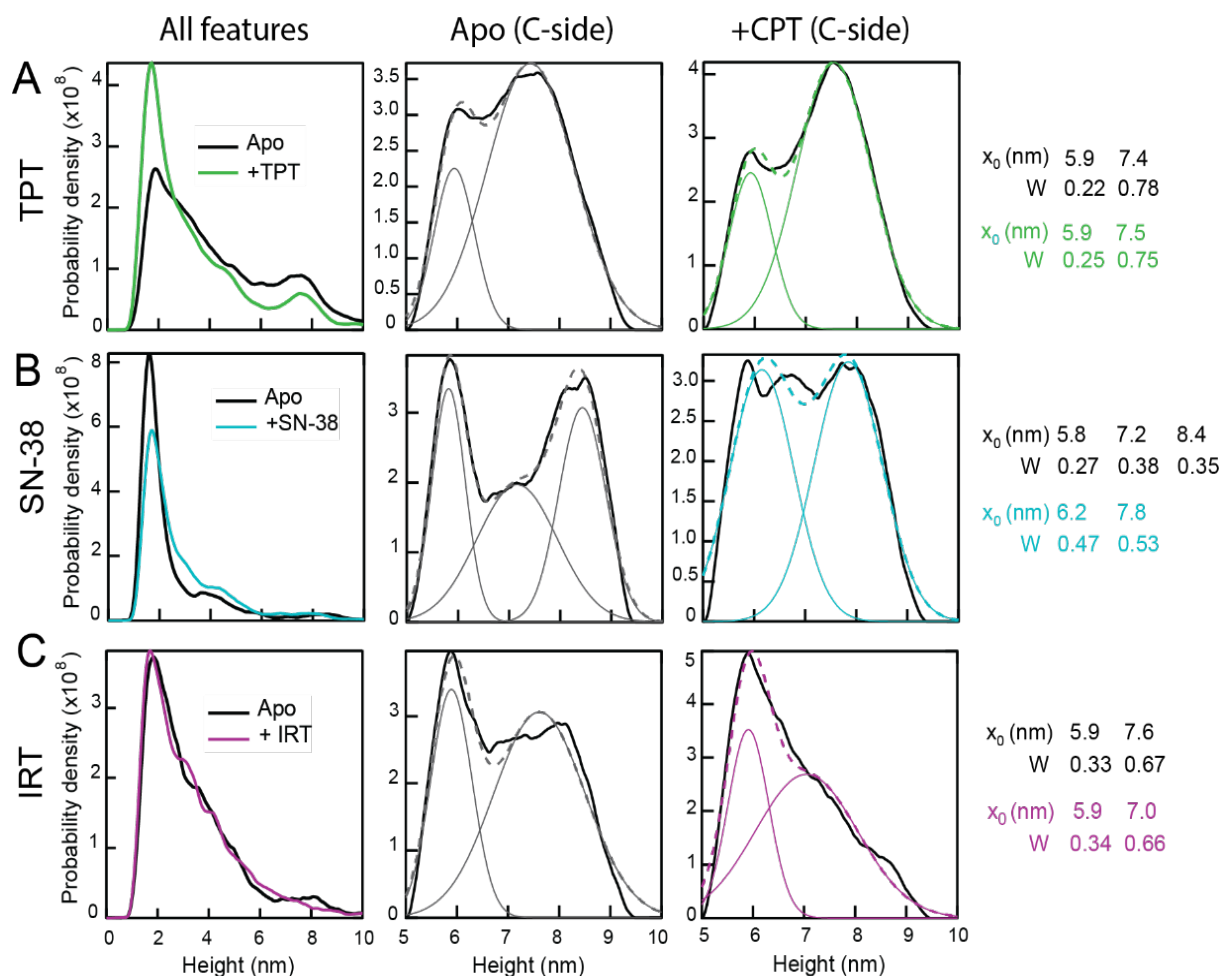


Figure S2.2 Histograms of Pgp in the presence of CPTs

Supplemental Figure S2.2. Histograms of Pgp + CPTs. (A) TPT, (B) IRT, and (C) SN-38. The right panels show histograms of all analyzed particles. The C-side apo data (middle panels; solid black line) is fitted using the Bayesian information criterion to determine the appropriate number of Gaussian distributions. Individual Gaussian distributions (solid gray lines) are summed together to form the model fit (dashed gray lines). Left panels display the +CPT conditions. Raw data is portrayed as a black line, with individual Gaussians (solid-colored lines) and summary fits (dashed colored lines) overlaid. All peak positions and weights are presented to the left of the graphs.

CHAPTER 3

EXPLORING THE MECHANISMS UNDERLYING PGP-MEDIATED TRANSPORT OF VINCA ALKALOIDS ³

³ Mensah, GAK; Schaefer, KG; Bartlett, M.G.; Roberts, A.G; King, G.M; Submitted to *Journal of Pharmaceutical Sciences*, October 26, 2023.

3.1 ABSTRACT

The efficacy of many cancer drugs is hindered by P-glycoprotein (Pgp), a cellular pump that removes drugs from cells. To improve chemotherapy, drugs capable of evading Pgp must be developed. Despite similarities in structure, vinca alkaloids (VAs) show disparate Pgp-mediated efflux ratios. ATPase activity and binding affinity studies show at least two binding sites for the VAs: high- and low-affinity sites that stimulate and inhibit the ATPase activity rate, respectively. The affinity for ATP from the ATPase kinetics curve for vinblastine (VBL) at the high-affinity site was 2- and 9-fold higher than vinorelbine (VRL) and vincristine (VCR), respectively. Conversely, VBL had the highest $K_m(\text{ATP})$ for the low-affinity site. The dissociation constants (K_{DS}) determined by protein fluorescence quenching were in the order $\text{VBL} < \text{VRL} < \text{VCR}$. The order of the K_{DS} was reversed at higher substrate concentrations. No significant Pgp conformational changes were observed for the VAs in the presence of 10 μM (using acrylamide quenching) and 150 μM (using acrylamide and Atomic Force Microscopy) drug concentrations. When 3.2 mM AMPPNP was present, 10 μM of either VBL, VRL, or VCR shifted Pgp to an open-outward conformation. In the presence of AMPPNP, 150 μM of VAs shifted the conformation of Pgp to an intermediate orientation, between opened inward and open-outward. However, the conformational shift induced by 150 μM VCR and AMPPNP was lesser than VBL and VRL. Additionally, STDD NMR studies revealed comparable groups in VBL, VRL, and VCR involved in Pgp-VAs interactions. Our results, when coupled with VAs-microtubule structure-activity relationship (SAR) studies, could lay the foundation for the development of effective next-generation VAs. A model that illustrates the intricate process of Pgp-mediated transport of the VAs is presented.

3.2 INTRODUCTION

Multidrug resistance (MDR) is a major obstacle hindering chemotherapy (Wang et al., 2017). The MDR phenotype has been observed in over 90% of cancer patients experiencing chemotherapeutic failures (Longley & Johnston, 2005). P-glycoprotein (Pgp), a well-studied member of the ATP binding cassette (ABC) superfamily, plays a pivotal role in the development of MDR (J. Zhang et al., 2013; Zhou, 2008). Pgp is a transmembrane transporter that expels a broad range of hydrophobic compounds, including drugs, from cells (Boumendjel et al., 2009). Overexpression and activation of Pgp significantly contribute to MDR in tumor cells (Robinson & Tiriveedhi, 2020). Interestingly, both intrinsic overexpression of Pgp in tumor cells and induction of acquired MDR by certain anticancer agents have been observed (Katayama et al., 2014; Nanayakkara et al., 2018). While numerous cellular signaling pathways facilitate Pgp overexpression, reports also detail cell-to-cell transfer of Pgp in cancer and non-cancer cells (Noack et al., 2016; Pasquier et al., 2011a; Pokharel et al., 2017). Multiple attempts have been made over the last three decades with limited success to address Pgp-mediated MDR (Amin, 2013; Nanayakkara et al., 2018). Although the use of specific Pgp inhibitors to enhance drug delivery showed early promise, their lack of specificity and high toxicity hindered success in clinical studies (Binkhathlan & Lavasanifar, 2013; Callaghan et al., 2014; Nanayakkara et al., 2018; C.-P. Wu et al., 2008). Another approach involves designing anticancer drugs that can bypass the transporter (Waghray & Zhang, 2017). However, this approach has not been fully realized due to a lack of comprehensive understanding regarding the structural and molecular mechanisms driving Pgp-mediated multidrug resistance. As a result, there has been considerable interest by pharmaceutical and regulatory agencies in understanding the structural and molecular factors that drive the Pgp-mediated transport of drugs (Keogh & Kunta, 2006).

Vinca alkaloids (VAs), a class of drugs initially derived from the *Catharanthus roseus* plant endemic to Madagascar (van Der Heijden et al., 2004), hold promise not only as anticancer agents but also for treating conditions like diabetes and hypertension due to their hypoglycemic properties (Tyagi et al., 2010). By binding to and disrupting mitotic spindle microtubule polymerization, VAs bring about cell cycle arrest at the M-phase and, coronally, cell deaths (Mhaidat et al., 2016; Moudi et al., 2013). The influence of Vas extends to other cellular processes, including metabolism and biosynthesis of amino acids, lipids, and nucleic acids (Vardanyan & Hruby, 2006). The VAs are one of the most-used class of anti-cancer agents due to their efficacy and include FDA-approved drugs such as vincristine (VCR), vinblastine (VBL), vinorelbine (VRL), vindesine (VDS), and vinflunine (VFL) (Dhyani et al., 2022; Moudi et al., 2013). The VAs possess similar dimeric structures comprising an indole nucleus (catharanthine) and a dihydroindole nucleus (vindoline) (Duffin, 2000) (Fig 1). While binding studies between VAs and isolated microtubules suggest similar affinities (Lobert et al., 1996; Ngan et al., 2000), disparities arise when assessing their interference with tubulin formation in vitro, which indicates an order of $VCR > VBL > VRL$ (Kruczynski et al., 1998; Lobert et al., 1996). In addition, the partition coefficient values of these drugs are not significantly different and could not account for the observed differences in their anticancer activities (Wishart et al., 2018; Zhigaltsev et al., 2005). The puzzle deepens when considering cellular retention and potency. VCR displays a slower release rate and notably higher cellular retention than VBL (Ferguson & Cass, 1985; Pierré et al., 1992; Rivera-Fillat et al., 1988). Similarly, VRL showcases a more significant volume distribution and enhanced cellular retention relative to VCR and VBL (Krikorian & Breillout, 1991). Interestingly, continuous treatment with either VBL or VCR yielded comparable cellular toxicity (Gout et al., 1984), implying a connection between cellular retention and anti-cancer potency. The discrepancy observed in the efficacy of

the VAs is likely due to the activity of transmembrane transporters such as Pgp (Barthomeuf et al., 2005; Iqbal et al., 2022) that differential reduces the cellular concentration of the Vas. In fact, despite sharing a basic structure and being similarly lipophilic, in vitro studies using Caco-2 cells indicate Pgp-mediated efflux rate of 11.9, 8.5, and 4.7 for VBL, VCR, and VRL, respectively (Lin et al., 2011). In the presence of Zosuquidar, a well-studied Pgp inhibitor, the transport rates for VBL, VCR, and VRL were reduced significantly to 6.5, 3.0, and 3.2, respectively (Lin et al., 2011). These findings highlight the crucial role Pgp plays in regulating the bioavailability of the VAs and, ultimately, their efficacy. Unfortunately, the underlying mechanisms driving the observed differences in Pgp-mediated transport rates of the VAs remain elusive. Any attempts aimed at developing the next generation of VAs without a comprehensive understanding of the underlying transport mechanism would remain a hit or miss. In this study, we provide the structural and biochemical basis for the Pgp-mediated transport of VAs. Drug-induced Pgp-mediated ATPase activities were investigated to identify plausible Pgp-ATP cooperativity since ATP hydrolysis drives the action of Pgp. The Pgp-drug affinity, another biochemical process that could dictate the activity of the protein, was investigated using drug-induced intrinsic tryptophan fluorescence quenching. STDD NMR was used to identify the functional groups in VAs likely involved in Pgp-drug interactions. Finally, drug-induced Pgp conformational changes were determined directly and indirectly by acrylamide quenching of Pgp intrinsic tryptophan fluorescence spectroscopy and atomic force microscopy (AFM), respectively. Our results, coupled with previously published results, were then used to build a simplified model that describes the structural mechanisms underlying Pgp-mediated transport of VAs.

3.3 EXPERIMENTAL REAGENTS AND METHODS

3.3.1 Chemicals and Reagents

Vincristine sulfate and vinblastine sulfate were procured from MP Biomedicals (Santa Ana, CA), while vinorelbine was sourced from Astatech Inc. (Bristol, PA). Adenylyl-imido diphosphate lithium salt (AMPPNP), N-acetyl-L-tryptophanamide (NATA), and ammonium chloride (NH₄Cl) were obtained from Sigma Aldrich (Milwaukee, WI). The detergent utilized for protein purification, n-dodecyl-β-D-maltoside (DDM), was obtained from EMD Millipore Corporation (San Diego, CA). Sodium orthovanadate (Na₃OVO₃) was procured from Enzo Life Sciences (Farmingdale, NY). Magnesium chloride (MgCl₂) and sodium chloride (NaCl) were sourced from J.T Baker (Center Valley, PA). Disodium Adenosine triphosphate (Na₂ATP), cholesterol, and Tris hydrochloric acid were acquired from Amresco (Solon, OH). Dithiothreitol (DTT) was purchased from Gold Biotechnology (St. Louis, MO). Acrylamide and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Calbiochem (San Diego, CA). Imidazole and ethylene glycol tetra-acetic acid (EGTA) were sourced from Alfa Aesar (Tewksbury, MA). Escherichia coli total lipid extract powder was procured from Avanti Polar Lipids Inc. (Alabaster, AL). Except otherwise stated, all other chemicals used in this study were purchased from Thermo Fisher Scientific (Waltham, MA).

3.3.2 Methods

3.3.2.1 Protein Expression and Purification

Pichia Pastoris engineered to overexpress his-tagged wild-type mouse Pgp (ABCB1A, MDR3) were cultured in glycerol-based media and further induced with methanol-based media to enhance the expression of proteins (Lerner-Marmarosh et al., 1999; Urbatsch et al., 1998) at the Bioexpression and Fermentation Facility (BFF), University of Georgia, Athens, GA. The cells

were harvested and subjected to a repeated cycle of freezing, blending, and thawing (Dunn & Wobbe, 2001). A two-step approach described previously (Bai et al., 2011; Lerner-Marmarosh et al., 1999; Wilt et al., 2017) was adopted with some modifications to purify the protein. Initially, an affinity chromatography technique using nickel-nitrilotriacetic acid (Ni-NTA) (ThermoFisher Scientific, Waltham, MA) was conducted, followed by ion-exchange chromatography with Whatman DE52 diethylaminoethyl cellulose (DEAE) resin (ThermoFisher Scientific). The Purified Pgp solubilized in DDM were concentrated using Amicon Ultra-15 100 kDa cut-off filters (EMD Millipore, Billerica, MA). An SDS/PAGE analysis was done as previously described (Lerner-Marmarosh et al., 1999) to assess the purity of the isolated protein. The purity was estimated to be greater than 95%. The concentration of the DDM-solubilized protein was determined with a Nanodrop (DeNovix, PHL), aliquots were made, and stored at -80°C.

3.3.2.2 Reconstitution of Pgp into liposomes

80% w/v Avanti E. coli total lipid extract and 20% w/v cholesterol were mixed in chloroform to a final concentration and volume of 10 mg/mL and 10 mL, respectively. The organic mixture was dried using Buchi Rotavapor, Model R-114 (Buchi, DE). The dried film was then re-suspended in 10 mL of rehydration buffer (0.1 mM EGTA and 50 mM Tris-HCl pH of 7.4) to create a solution of liposomes with various sizes. Resuspension of the liposomes was facilitated by a freeze-thawed cycle using liquid nitrogen. Liposomes of varying sizes created by this approach were extruded at least 11x through a 400 nm cut-off filter installed on a LIPEX extruder instrument (Northern Lipids, Burnaby, British Columbia, Canada) to create homogeneously sized liposomes. Dynamic Light Scattering (DLS) was used to assess the sizes and uniformity of the extruded liposomes using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The DLS

chromatograms were analyzed with Zetasizer 7.03 software and showed homogenously sized liposomes with an average size of 250 nm.

Pgp solubilized in detergent was dialyzed against HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 2 mM DTT, pH 7.4) for 2 hours to remove residual detergents. The dialyzed Pgp was mixed with previously extruded liposomes to create a ratio of 0.16 mgml⁻¹ liposomes per μM⁻¹ protein. The mixture was incubated on ice for 1 hour to promote protein integration into the liposomes before being dialyzed again on HEPES buffer for 2 hours to remove excess detergents and further enhance protein integration into the liposomes to form proteo-liposomes. The concentration of the proteo-liposomes was determined using a Bio-Rad DC Protein assay kit II, and aliquots were stored at -80°C in HEPES buffer, pH 7.4.

3.3.2.3 Pgp-mediated ATPase Activity

As previously described, the Chifflet method was used to determine vanadate-sensitive Pgp-mediated ATP hydrolysis (Chifflet et al., 1988). ATP hydrolysis generates free inorganic phosphates (*Pi*) as by-products (Scian et al., 2014). In the Chifflet assay, the rate of ATP hydrolysis is indirectly detected by estimating the turnover of inorganic phosphate. *Pi* produced form complexes with molybdenum, which characteristically has a high absorbance at 850nm. The ligand-induced ATPase activity assays for VBL, VCR, and VRL were carried out in Chifflet buffer (50 mM Tris-HCl, 150 mM NH₄Cl, 5 mM MgSO₄, 0.02% NaN₄, pH 7.4) with 250 nM of Pgp reconstituted into liposomes (Chifflet et al., 1988; Ledwitch, Gibbs, et al., 2016). Absorbance was detected on a Flexstation (III) spectrometer (Molecular Devices, Sunnyvale, CA) in a costar 96-well plate. If monophasic, the generated Pgp-mediated ATP hydrolysis kinetic curves are fitted to a modified Michaelis-Menten equation that accounts for the basal activity ([Eqn1](#)) (Segel, 1993).

The fitting was done with Python scripts developed in Igor Pro 6.2 software (Wavemetrics, Tigard, OR) to generate the kinetic parameters.

$$V = \frac{V_{\max}[S]}{K_m + [S]} + V_{\text{basal}} \quad (1)$$

Where v is the rate of ATP hydrolysis, $V_{\max}$ is the maximum rate of ATP hydrolysis, $[S]$ is the substrate concentration, K_m is the Michaelis-Menten constant, and V_{basal} is the basal ATPase activity in the absence of drugs.

For biphasic ATP hydrolysis showing a second substrate inhibition, the kinetic curves were fit into [Eqn2](#) below to determine the kinetic parameters: inhibitory constant (K_i), $V_{\max}$, and K_m .

$$V = \frac{V_{\max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} + V_{\text{basal}} \quad (2)$$

3.3.2.4 Drug–Pgp Affinity Determined by Intrinsic Tryptophan Fluorescence Quenching

Tryptophan residues in a protein absorb and emit significantly around 280 nm and 330 nm, respectively (Nguyen et al., 2020). The interactions with ligands can induce changes in the local environment that house the tryptophan residues, thereby altering the fluorescence intensity of the protein (Yammine et al., 2019). Quenching of tryptophan residues in proteins has been exploited in earlier studies to determine the binding affinity of ligands such as drugs and nucleotides (Ledwith, Gibbs, et al., 2016; R. Liu et al., 2000; Sharom et al., 2003). In this study, the protein fluorescence emission was done using an Olis DM 45 spectrophotometer (Olis Corporation, Bogart, CA). A 10 nm band-pass filter was placed on the excitation and emission paths of the spectrophotometer to remove Rayleigh bands from the fluorescence emission spectra. The investigation was conducted on samples containing Chifflet buffer (pH 7.4), 1 μM of P-gp

reconstituted into liposomes, and 2 mM DTT (Ledwitch, Gibbs, et al., 2016). The resultant ligand-induced fluorescence quenching data was corrected ($F_{\text{corrected}}$) for background, dilution, and inner filter effects with the [Eqn3](#) below (Lakowicz, 2011; Sharom et al., 2003).

$$F_{\text{corrected}} = (F - B)10^{\frac{(\epsilon_{\text{ex}}b_{\text{ex}} + \epsilon_{\text{em}}b_{\text{em}})[Q]}{2}} \quad (3)$$

The measured protein fluorescence at 330 nm is represented as F , while B signifies the background, and $[Q]$ denotes the ligand concentration (quencher). The extinction coefficient of the ligand for excitation and emission wavelengths is represented as ϵ_{ex} and ϵ_{em} , respectively. The extinction coefficients for the VAs were determined using previously described method (Jeewantha et al., 2017; Reddy & Reddy, 2012). The extinction coefficient (ϵ_{ex}) values at 280 nm for VBL, VRL, and VCR were determined to be $4.19 \text{ mM}^{-1}\text{cm}^{-1}$, $9.28 \text{ mM}^{-1}\text{cm}^{-1}$, and $4.25 \text{ mM}^{-1}\text{cm}^{-1}$, respectively. The path lengths along the excitation and emission axes are represented by b_{ex} and b_{em} , respectively. The estimated emission extinction coefficients (ϵ_{em}) at 330 nm for VBL, VRL, and VCR were $0.631 \text{ mM}^{-1}\text{cm}^{-1}$, $2.79 \text{ mM}^{-1}\text{cm}^{-1}$, and $0.085 \text{ mM}^{-1}\text{cm}^{-1}$, respectively. Two main mechanisms typically underlie ligand-induced protein fluorescence quenching: static and dynamic quenching (Lakowicz, 2011). In static quenching, ligands form complexes with the protein, while dynamic fluorescence quenching arises from random collisions between the protein and ligand (Lakowicz, 2011). The effect of two or more different temperatures on the fluorescence quenching curve was used to identify the predominant quenching mechanism present (Lakowicz, 2011). When dynamic quenching is predominant, elevated temperatures increase collision frequency, resulting in higher K_{sv} values (Lakowicz, 2011). In contrast, for static quenching, higher temperatures decrease the residency time of the ligand-protein complex, yielding lower K_{sv} values (Lakowicz, 2011). The VAs showed a biphasic curve, exhibiting static quenching and dynamic

mechanisms at relatively low and high concentrations, respectively. However, in the presence of AMPPNP, the VAs showed a monophasic quenching curve, which was determined to be primarily static (results not shown). In the case of monophasic fluorescence quenching, the corrected fluorescence ($F_{\text{corrected}}$) quenching curves were fitted to a Stern-Volmer equation below ([Eqn4](#)) to determine the Stern-Volmer constant (K_{sv}) values.

$$F_{\text{corrected}} = \frac{F_{\text{o(corrected)}}}{1+K_{\text{sv}}[Q]} + F_{\text{unquenched}} \quad (4)$$

Here, the $F_{\text{o(corrected)}}$ represents the fluorescence in the absence of a quenching ligand, $F_{\text{unquenched}}$ is an offset related to unquenched fluorescence, and $[Q]$ is the concentration of the ligand. Conversely, fluorescence quenching curves showing biphasic characteristics were fitted to a modified Stern-Volmer equation ([Eqn 5](#))

$$F_{\text{corrected}} = \frac{F_{\text{o,L}}}{1+K_{\text{sv,H}}[Q]} + \frac{F_{\text{o,H}}}{1+K_{\text{sv,L}}[Q]} + F_{\text{unquenched}} \quad (5)$$

The amplitudes of protein fluorescence at low and high ligand concentrations are denoted as $F_{\text{o,L}}$, and $F_{\text{o,H}}$, respectively. Correspondingly, the K_{sv} values for the low and high ligand concentrations are represented as $K_{\text{sv,H}}$ and $K_{\text{sv,L}}$, respectively.

3.3.2.5 Drug-induced Pgp Conformational Changes Probed by Acrylamide Quenching

Acrylamide is a small polar quencher that can effectively quench the fluorescence emitted by tryptophan residues within proteins through collision (Lakowicz, 2011; R. Liu et al., 2000; Strambini & Gonnelli, 2010). Data from prior studies suggests that proteins can undergo alterations in their conformation when they interact with ligands (Cioni & Strambini, 1998). These conformational changes could either occlude or expose the tryptophan residues in the protein, leading to changes in the solvent-accessible tryptophan residues (Russell & Sharom, 2006; Sharom et al., 2003; Sonveaux et al., 1999). As acrylamide is unable to penetrate the hydrophobic core of

the protein easily, alterations in the protein's fluorescence can provide insights into potential modifications in the protein's tertiary structure caused by ligand interactions (Eckford & Sharom, 2008; R. Liu et al., 2000; Zoghbi et al., 2017). In this study, as acrylamide is titrated against the sample solution, the fluorescence intensity is measured at 330 nm, following excitations at 280 nm. The sample solution consists of 1 μ M Pgp reconstituted into liposomes in Chifflet buffer supplemented with 2 mM DTT. The fluorescence intensities were corrected with [Eqn3](#), and a Stern-Volmer plot of $F_{o(\text{corrected})}/F_{(\text{corrected})}$ against the acrylamide concentration was generated. The slope of the Stern-Volmer curve is a function of the degree of quenching, and it is related to the K_{sv} value by [Eqn6](#) below (Lakowicz, 2011):

$$\frac{F_{o(\text{corrected})}}{F_{(\text{corrected})}} = 1 + K_{sv}[Q] \quad (6)$$

3.3.2.6. Saturation Transfer Double Difference (STDD) NMR

STDD NMR is a powerful ligand-focused technique used to study ligand-receptor interactions and map epitopes (Roberts et al., 2011; Viegas et al., 2011). The principle of STD NMR involves selectively saturating the protons in a receptor with a long, low-power pulse (Roberts et al., 2011). When a ligand binds to the receptor, protons within approximately 5 Å from the saturated protein experience significant saturation transfer from the receptor (Xu et al., 2021). Upon dissociation, the saturation transferred to the ligand is detected as saturation transfer difference (STD). The degree of saturation transferred inversely corresponds to the ligand-receptor distance and reflects the strength of the interaction. Since many Pgp substrates are highly lipophilic, nonspecific interactions between ligands and liposomes can interfere with observed STD NMR spectra. The STD NMR spectrum of the drug in the presence of liposomes was subtracted from the STD ^1H NMR spectrum of the ligand with proteins reconstituted into liposomes to account for potential STD contributions from ligand-liposome interactions. This subtraction leads to the generation of

saturation transfer double difference (STDD) spectrum (Claasen et al., 2005; Haselhorst et al., 2007). This approach was employed to investigate the functional groups of VBL, VCR, and VRL potentially involved in Pgp-drug interactions. All NMR experiments were conducted at 25°C (unless stated otherwise) on a Varian INOVA 600 MHz NMR spectrometer equipped with a ^1H -detect broadband probe {HX}. Data analyses were performed with [iNMR](#) software (Nucleomatic, Molfetta, Italy), Igor Pro 6.2, and [Mnova 14.2.0](#) (Mastrelab Research S.L, Santiago de Compostela, Spain). The STDD NMR procedure in this study was performed as described (Venkitakrishnan et al., 2012). 100 mM potassium phosphate buffer [80% D_2O , 20% ddH $_2\text{O}$, pH 7.4] containing 1 μM Pgp reconstituted into liposomes, and 1 mM of either VBL, VRL or VCR was prepared. Control samples were similarly prepared with liposomes instead of Pgp-reconstituted liposomes. The WATERGATE gradient pulse sequence was used to suppress water signals that would have otherwise dwarfed the signals from the ligand (M. L. Liu et al., 1998; Piotto et al., 1992). Selective excitation and saturation of the protein were achieved using a 50 ms train of Gaussian-shaped-selective pulses for 2s, followed by a delay of 5s to enable relaxation (Mayer & Meyer, 2001; Viegas et al., 2011). STD NMR spectra were obtained through phase cycling by alternating Pgp irradiation resonance between -1.5 ppm (on resonance) and 42 ppm (off-resonance) for 512 scans. Subtracting the off-resonance from the on-resonance spectra generated the STD ^1H NMR spectrum. Control experiments comprising liposomes and drugs were conducted under the same conditions and subtracted from the ^1H STD NMR spectrum of the reconstituted Pgp sample with the drug, yielding the STDD ^1H NMR spectrum. The observed difference (ΔI) directly reflects the interaction strength between drug functional groups and the protein. The STDD amplification factor was calculated using [Eqn7](#) (Ledwitch, Barnes, et al., 2016; Mayer & Meyer, 2001).:

$$STDD \text{ Amplification Factor} = \frac{[l] \Delta I}{[P] I_0} \quad (7)$$

Where I_0 represents the amplitude of the ^1H NMR peaks, $[l]$ denotes the ligand concentration, and $[P]$ is the protein concentration.

3.2.7 Atomic Force Microscopy

Atomic force microscopy (AFM) is a robust technique initially developed to examine the physical characteristics of solid-state materials by directly probing their surfaces with a mechanical probe (Müller et al., 2021). This technique has been adopted in earlier studies to analyze protein dynamics and structure, facilitating the identification of ligand-induced conformational changes in proteins (Nguyen et al., 2020; Sigdel et al., 2018; Vahabi et al., 2013). In the present study, a solution of Pgp reconstituted into liposomes (proteoliposomes) was diluted in imaging buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$; pH 7.4) to about 100 nM. About 90 μL of this solution was deposited onto a freshly cleaved mica surface (Grade v1; Ted Pella, Redding, CA) and then incubated at 25°C for approximately 30 minutes. The incubation led to the rupture of proteoliposomes, forming a planar lipid bilayer atop the mica surface. The AFM imaging of the EC-side and C-side domains of Pgp protruding over the lipid bilayer was carried out as previously described (Sigdel et al., 2018). The samples were rinsed with 90 μL of buffer at least 5 times using a buffer exchange technique. AFM imaging of the EC-side and C-side domains of Pgp protrusions above the lipid bilayer was performed as previously described (Sigdel et al., 2018). For each of the selected VAs under study, AFM imaging of the ligand-free Pgp (apoPgp) was performed first, followed by the addition of 150 μM of either VBL, VRL, or VCR. AFM imaging was repeated upon the addition of ligands such that a nominally similar area of the sample was captured. Only AFM images that could be positively assigned as either EC or C-side domain features of Pgp were analyzed. The AFM imaging was obtained in an imaging buffer at 25°C in tapping mode with

biolever mini tips installed on a commercial instrument (Asylum Research Cypher, Santa Barbara, CA). Tips (BL-AC40TS, Olympus, Tokyo, Japan) with an estimated tip-sample force < 100 pN were used to minimize the probability of protein distortion. The generated AFM images were subsequently analyzed using custom software developed in Igor Pro 7 (Wavemetrics, Portland, OR) as previously done (Marsh et al., 2018; Sigdel et al., 2018). The smoothed histograms were constructed in Igor Pro 7 using kernel density estimation with Epanechnikov kernels (Chu et al., 2017).

3.4 RESULTS

3.4.1 *Pgp-mediated ATPase activity*

The Pgp catalytic cycle is an energy-dependent process (Sauna & Ambudkar, 2007; Sharom, 2011b) that requires at least one ATP molecule per VA molecule transported (Shalinsky et al., 1993). This study assessed a potential coupling effect of ATP hydrolysis on Pgp-mediated transport of VAs. Figure 2 shows the effect of selected VAs on Pgp-mediated ATP hydrolyses. Panels 2A, 2B, and 2C show the kinetic curves of drug-induced Pgp-mediated ATPase activity in the presence of increasing concentrations of VBL, VRL, and VCR, respectively. The observed basal activities of Pgp in the absence of VBL, VCR, and VRL (dashed lines) were similar, averaging $477 \pm 22 \text{ nmolmin}^{-1}\text{mg}^{-1}$, highly consistent with previous estimates (Wilt et al., 2017). Drug-induced Pgp-mediated ATP-hydrolyses kinetic curves deduced for all the tested compounds were biphasic, which suggests that at least two distinct rate-limiting steps or mechanisms are involved. The two phases consist of substrate stimulation at lower drug concentrations ($< 20 \mu\text{M}$), reaching maximum activation of 35%, 18%, and 16% over the basal activity level for VBL, VRL, and VCR, respectively. ATPase activation was followed by substrate inhibition at higher concentrations, reducing the maximum activity of VBL, VRL, and VCR by 51%, 27%, and 24%, respectively. Previous studies have observed Similar Pgp binding modes for the VAs (Garrigos et al., 1997; Martin et al., 2001; Safa, 2004; Tamai & Safa, 1990). The kinetic curves generated were fit to a modified Michaelis-Menten equation for biphasic substrate inhibition ([Eqn2](#)) to determine the kinetic parameters V_{max} , K_m , and K_i values. The K_m and K_i deduced for VBL (Fig. 2A) were $1.78 \pm 2.34 \mu\text{M}$ and $24.17 \pm 6.12 \mu\text{M}$, respectively, which agree with a previous study where a biphasic kinetic curve was observed (Aanismaa & Seelig, 2007). On the contrary, in another study where only the inhibitory phase of the curve was observed, probably due to the concentration of

the drug used, the $K_m(\text{ATP})$ induced by VBL was substantially different (Sharom et al., 1995). The apparent $V_{\max}$ extracted from the VBL-induced ATPase kinetics curve was $601 \pm 30 \text{ nmolmin}^{-1}\text{mg}^{-1}$. The K_m , K_i , and $V_{\max}$ values generated from the VRL-induced ATPase kinetic fit (Fig 2B) were $5.12 \pm 3.87 \text{ }\mu\text{M}$, $8.79 \pm 4.76 \text{ }\mu\text{M}$, and $590 \pm 21 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively. For VCR (Fig. 2C), the estimated K_m and K_i values were $17.04 \pm 6.50 \text{ }\mu\text{M}$ and $7.40 \pm 3.26 \text{ }\mu\text{M}$. The associated $V_{\max}$ for the VCR was $558 \pm 33 \text{ nmolmin}^{-1}\text{mg}^{-1}$. Overall, these results are congruent with previous studies that suggest that vinca alkaloids act as both Pgp substrate and inhibitor of Pgp-coupled ATP hydrolysis depending on the availing concentrations (Aanismaa & Seelig, 2007; Cisternino et al., 2004). However, unlike verapamil, which almost completely inhibits Pgp activity at $50 \text{ }\mu\text{M}$ (Ambudkar et al., 1997) with an average K_i of $1.9 \pm 0.5 \text{ }\mu\text{M}$ (Ledwitch, Barnes, et al., 2016), the degree of inhibition by VAs is relatively mild and may fall outside of physiological relevant dose (Yau et al., 1985; Young et al., 2006). In addition, the VAs have relatively high non-target toxicity, making their usage as a potential inhibitor untenable. These results are summarized in Table 1.

3.4.2 Pgp-Ligand Binding Affinity Probed by Protein Fluorescence Quenching

Fluorescence changes in proteins caused by drug interactions with tryptophan residues in or near the drug-binding pocket can be a valuable tool for assessing drug-protein binding affinity (Lakowicz, 2011; Yammine et al., 2019). As previously done (Gayer et al., 2020; R. Liu et al., 2000; Sharom et al., 2003), this technique was used to determine the apparent binding affinity of the VAs to Pgp. Figure 3 shows the effect of the VAs on Pgp fluorescence. Panels 3A, 3C, and 3E represent the spectra of VAs-induced Pgp emission decay observed between 300 nm to 500 nm. Pgp fluorescence emission spectra showed a maximum at 330 nm following excitation at 280 nm. The upper and lower thick black lines show the maximum and minimum protein fluorescence observed in the absence and presence of $150 \text{ }\mu\text{M}$ of drugs, respectively. The grey lines between

the maximum and minimum spectra show the protein fluorescence spectra at 0 μM and 150 μM concentrations. The maximum protein fluorescence observed at 330 nm decreased by approximately 55%, 40%, and 32% in the presence of 150 μM of VBL, VRL, and VCR, respectively. In the presence of AMPPNP, 150 μM of VBL induced about 34% decrease in protein fluorescence. Similarly, VCR and VRL decreased the protein's fluorescence by 30% and 38%, respectively, in the presence of saturating AMPPNP (results not shown). The amplitudes of Pgp fluorescence were monitored at 330 nm as VBL, VRL, and VCR were titrated against the protein. The resulting fluorescence amplitudes were adjusted for the inner filter effect using [Eqn3](#), and the corrected fluorescence amplitudes, $F(\text{corrected})$, plotted against the increasing concentration of VBL (Fig 3B), VCR (Fig 3D), and VRL (Fig. 3F) in the absence (closed squares) and presence of (opened squares, dashed lines) saturating AMPPNP. The Pgp fluorescence quenching curves for the VAs were biphasic: a high-affinity phase at relatively low drug concentrations and low affinity at high concentrations. The K_{sv} values were determined by fitting the curve to [Eqn5](#) for a biphasic binding curve. When the vinca alkaloids were titrated against the protein at a higher temperature (37°C) and compared to the results at room temperature, the $K_{sv,H}$ values observed at relatively lower drug concentrations ($< 20 \mu\text{M}$) decreased significantly, while the $K_{sv,L}$ value decreased substantially. These temperature-dependent changes in K_{sv} values suggest that the predominant quenching mechanisms at the high- and low-affinity binding sites are static and dynamic, respectively (Lakowicz, 2011). In the presence of saturating AMPPNP, the generated Pgp fluorescence quenching curves for the VAs were monophasic and were fit to [Eqn4](#) to determine the apparent K_{sv} values. The K_{sv} values for VBL, VRL, and VCR in the presence of saturating AMPPNP decreased significantly at a higher temperature (37°C), suggesting a predominantly static quenching mechanism. For a static quenching mechanism, the K_{sv} values correlate with the

equilibrium binding constant. The $K_{D,H}$ and $K_{D,L}$ values for Pgp-drug interactions were determined from their respective $K_{sv,H}$ and $K_{sv,L}$ values obtained from the protein fluorescence curves. The K_{sv} and K_D values for Pgp-VAs interactions are summarized in Table 2. The $K_{sv,H}$ and $K_{D,H}$ values for VBL were $0.849 \pm 0.06 \mu\text{M}^{-1}$ and $1.18 \pm 0.07 \mu\text{M}$, respectively, which are within the range previously reported (Martin et al., 1999, 2001; Sharom, 1997; Sharom et al., 1999). The $K_{sv,L}$ and $K_{D,L}$ for VBL were $0.015 \pm 0.004 \mu\text{M}^{-1}$ and $67.67 \pm 5.08 \mu\text{M}$, respectively. In the presence of AMPPNP, a monophasic fluorescence binding curve was observed for VBL, resulting in K_{sv} and K_D values of $0.052 \pm 0.006 \mu\text{M}^{-1}$ and $19.26 \pm 2.25 \mu\text{M}$, respectively. Similarly, the $K_{sv,H}$ and $K_{sv,L}$ values deduced for Pgp-VRL interactions were $0.393 \pm 0.087 \mu\text{M}^{-1}$ and $0.032 \pm 0.01 \mu\text{M}^{-1}$, respectively. The corresponding $K_{D,H}$ and $K_{D,L}$ values for VRL were $3.84 \pm 0.61 \mu\text{M}$ and $30.96 \pm 5.16 \mu\text{M}$ for the high- and low-affinity binding sites on Pgp. The K_{sv} and K_D values deduced from the interaction between VRL and Pgp in the presence of saturating AMPPNP were $0.040 \pm 0.019 \mu\text{M}^{-1}$ and $25.00 \pm 2.49 \mu\text{M}$, respectively. The Pgp-VCR interactions also exhibited two distinct binding affinities, with $K_{D,H}$ and $K_{D,L}$ values of $2.55 \pm 0.16 \mu\text{M}$ and $24.89 \pm 5.64 \mu\text{M}$, respectively. These K_D values were derived from the $K_{sv,H}$ and $K_{sv,L}$ values of $0.393 \pm 0.087 \mu\text{M}^{-1}$ and $0.040 \pm 0.003 \mu\text{M}^{-1}$, respectively. These results agree with previously reported data suggesting a high and low-affinity uptake of VCR by Pgp-containing membrane vesicles (Tamai & Safa, 1990). AMPPNP shifted VCR into a monophasic binding curve with K_{sv} and K_D values of $0.104 \pm 0.038 \mu\text{M}^{-1}$ and $9.58 \pm 3.51 \mu\text{M}$, respectively.

3.4.3 Ligand-induced Conformational Changes in Pgp Probed by the Acrylamide Quenching of Protein Fluorescence.

Pgp-mediated transport of ligands is believed to be modulated by conformational changes in the protein (Dawson et al., 2007; Lakowicz, 2011; Sonveaux et al., 1999; A. Ward et al., 2007).

Ligand-induced conformational changes in proteins have been estimated previously by probing the changes in relative exposure of solvent-accessible tryptophan residues within the protein (Akbar et al., 2016; Gibbs et al., 2018; Jazaj et al., 2019). In this study, acrylamide quenching of readily accessible tryptophan residues was used to investigate whether ligand-induced conformational changes in the protein modulate Pgp-mediated transport of the VAs. Quenching is typically most pronounced when Pgp is in an open-inward conformation, where the tryptophan residues within or near the binding pockets are easily accessible by the surrounding solvent (R. Liu et al., 2000; Moller & Denicola, 2002). Conversely, quenching is minimal in the open-outward conformation due to the shielding of the solvent-accessible tryptophan residues. Figure 4 shows the Stern-Volmer plots illustrating the quenching of Pgp fluorescence by acrylamide in the presence of the selected VAs under study. The slopes obtained from these curves, which are equivalent to the K_{sv} values, provide an estimate of the extent of quenching. Changes in the estimated K_{sv} value indicate a shift in the protein's tertiary structure, influencing the number of solvent-accessible tryptophan residues. Figure 4A presents the Stern-Volmer plot of NATA (Fig. 4A, opened circle) and Pgp (Fig. 4A, opened squares) in the absence of ligands (apoPgp). The K_{sv} values were determined by fitting the curve into [Eqn6](#) after accounting for the inner filter effect, dilution, and background with [Eqn3](#). The estimated K_{sv} values for NATA (a free analog of tryptophan residue) and apoPgp were $31.54 \pm 2.19 \text{ M}^{-1}$ and $4.17 \pm 0.15 \text{ M}^{-1}$, respectively. Had all the tryptophan residues in the protein been solvent-accessible, the estimated K_{sv} value of apoPgp would have been expected to match that of NATA. However, since acrylamide quenching of the tryptophan residues are only feasible when the distance between the fluorophore and the quencher is $< 3 \text{ \AA}$ (Strambini & Gonnelli, 2010), these are congruent with earlier report suggesting that most of the tryptophan residues in Pgp are buried within the hydrophobic region of the protein and are

not readily solvent accessible (Swartz et al., 2020). Previous reports indicate that only 3 of the 11 tryptophan residues in Pgp are likely solvent-accessible (Swartz et al., 2013, 2020). Note that the solved x-ray structure of mammalian apoPgp and other analogous bacterial transporters showed the protein in an open-inward conformation (Esser et al., 2017; Jin et al., 2012; J. Li et al., 2014; Ye & Berg, 2004). Subsequently, it was assumed that the K_{sv} value of apoPgp represents the protein in an open-inward conformation, where the two nucleotide-binding domains (NBDs) are apart. In the presence of saturating AMPPNP, the K_{sv} value of Pgp decreased to $1.99 \pm 0.041 \text{ M}^{-1}$ (Fig 4B, closed triangle), suggesting a shift to the open-outward conformation where the fluorescence residues are relatively occluded from the bulk solvent. This inference is based on reports showing an open-outward conformation in x-ray crystal and Cryo-Em structures of Pgp in the presence of nucleotides (Kim & Chen, 2018; Moeller et al., 2015; Szewczyk et al., 2015) (Kim & Chen, 2018). Stern-Volmer's plots of Pgp in the presence of 10 μM of VBL, VRL, and VCR are presented in panels 4C, 4E, and 4G. These plots (as well as 4D, 4F, and 4G) compare the fluorescence quenching in the absence (closed square) and presence (opened square) of 3.2 mM AMPPNP. The estimated K_{sv} value deduced from the Stern-Volmer plots of Pgp in the presence 10 μM of either VBL, VRL, or VCR were $3.87 \pm 0.16 \text{ M}^{-1}$, $3.91 \pm 0.35 \text{ M}^{-1}$, and $4.02 \pm 0.15 \text{ M}^{-1}$, respectively, suggesting that the binding of VAs to Pgp at low concentrations does not significantly influence the conformation of the protein. In the presence of AMPPNP, however, the K_{sv} value obtained for the VAs at 10 μM decreased considerably to $1.84 \pm 0.032 \text{ M}^{-1}$, $2.04 \pm 0.049 \text{ M}^{-1}$ $2.58 \pm 0.069 \text{ M}^{-1}$ for VBL, VRL, and VCR, respectively. In the presence of 150 μM of VBL, VRL, and VCR, the K_{sv} values deduced from the Stern-Volmer's plot of Pgp fluorescence were $3.94 \pm 0.19 \text{ M}^{-1}$, $4.10 \pm 0.05 \text{ M}^{-1}$, and $4.30 \pm 0.12 \text{ M}^{-1}$, respectively. The K_{sv} values induced by 150 μM VBL, VRL, and VCR decreased significantly to $2.75 \pm 0.047 \text{ M}^{-1}$, $2.85 \pm 0.08 \text{ M}^{-1}$, $3.6 \pm 0.088 \text{ M}^{-1}$,

respectively, in the presence of AMPPNP. These results, taken together, suggest that the binding of nucleotides may be required to induce the conformational shift in Pgp necessary for transport.

3.4.4 Identification of Ligand-Receptor Interactions by STDD NMR

STD NMR is a robust spectroscopic technique used to study interactions between macromolecules like proteins and ligands in solution (Monaco et al., 2017; Walpole et al., 2019; Xu et al., 2021). In this study, we employed the STDD NMR technique to investigate the specific moieties on VAs that interact with the receptor, Pgp. Figure 5 displays the STDD NMR spectra, highlighting the protein-ligand interactions between VAs and Pgp. Panels 5A, 5C, and 5E show the ^1H 1D STDD NMR spectra for VBL, VRL, and VCR, respectively. STDD spectra for the VAs were compared with their respective 1D ^1H spectra (data not shown) to identify and assign the peaks that showed significant STDD. The 1D ^1H spectra observed for the VAs under study were consistent with previously reported studies (Dubrovay et al., 2013; Hada et al., 2013; Kumar et al., 2013). Due to their relatively larger molecular sizes, the VAs exhibited substantial STDD NMR signals from various functional groups. Interestingly, identical moieties on VBL, VRL, and VCR displayed significant STD NMR signal amplitudes, with the highest peaks originating from the methyl groups. Specifically, signals from C₄₂, C₄₁, and C₅₆ (as shown in Fig. 1) displayed the highest amplitudes for VBL, VRL, and VCR, respectively. The data obtained from the STDD NMR spectrum were fit to [Eqn7](#) to calculate the amplification factor, which normalizes the STDD amplitudes and accounts for multiple protons giving rise to a single peak. Category plots of STDD amplification factors estimated for VBL, VRL, and VCR are presented in panels 5B, 5D, and 5F, respectively. On average, VBL exhibited the highest STDD amplification factors per peak, followed by VRL. C₂₇ notably displayed the highest amplification factor for the three drugs, indicating its potentially critical role in the interaction between VAs and Pgp. In addition, other

protons arising from C₅₆, C₂₇, C₅₉, C₂₅, C₃₀, and C₂₆ (Fig. 5; see Fig. 1) showed significant amplification amplitude for the Pgp-VAs interactions.

3.4.5 Analysis of the impact of VBL, VRL, and VCR on the height distribution of Pgp determined by AFM imaging.

The catalytic function of Pgp relies significantly on its conformational transition from an open-inward orientation (open towards the cytosolic region) to an open-outward orientation (open to the extracellular environment) (Zoghbi et al., 2017). AFM is a high-resolution imaging technique that has been used to explore ligand-induced conformational changes in Pgp (Pinho & Piedade, 2013; Schaefer et al., 2023; Sigdel et al., 2018). Ligands efficiently transported by the protein typically facilitate conformational changes that reposition the protein into an open-outward configuration (Loo et al., 2003). AFM imaging was employed to investigate drug-induced conformational shifts that might influence Pgp-mediated transport of the VAs. While techniques like X-ray crystallography and cryogenic Electron Microscopy (cryo-EM) have contributed significantly to our understanding of this protein, they rely on the average coordinates of atoms to determine the structures of biological molecules (Kondrashov et al., 2007). In contrast, AFM imaging has the advantage of sampling through various conformations of a biological molecule, allowing the identification of conformational changes in individual molecules (M. Li et al., 2017).

Figure 6 provides insights into ligand-induced conformational changes in Pgp determined by AFM imaging. The identification of C-domains and EC-domains within the lipid bilayer was achieved by measuring the particle heights as in previous work (Schaefer et al., 2023; Sigdel et al., 2018). The C-side features were identified based on heights ranging from 55 to 90 Å. On the other hand, heights between 10 Å and 35 Å were attributed to EC-side features (Fig. 6A). C-side-specific antibodies for Pgp have been employed in a previous study to ensure a robust differentiation of the

C-side from the EC-side (Schaefer et al., 2023). Smoothed histograms demonstrating height shifts in the C-side features of Pgp in the absence (Fig. 6B-D; black lines) and presence of saturating VBL (Fig 6B), VRL (Fig 6C), and VCR (Fig 6D) are shown in Fig. 6. Analysis focused on C-side features due to the larger conformational dynamics on that side of the protein. To address potential batch-to-batch variations in the samples being imaged, we initiated the AFM imaging process by capturing images of Pgp without ligands. Subsequently, test compounds were introduced to the sample, and the imaging procedure was repeated. This approach ensures that any observed shift in features occurs within a reasonably consistent distribution of particles. The AFM imaging data were analyzed using a comprehensive model selection criterion (See Supporting Information Figure S1) and shifts in the tallest population were closely examined.

The sample size (N) of C-side features of apoPgp analyzed prior to the addition of VBL was 707, with the average height centered around $69 \pm 9 \text{ \AA}$ ($\pm\sigma$). The addition of 150 μM of VBL ($N=609$) shifted the height distribution slightly to the left, with the mean height reducing to $68 \pm 9 \text{ \AA}$ (Fig 6E, orange). Analysis of the C-side features was repeated for VRL and VCR. For VRL, apoPgp ($N = 1241$) had a mean height of $68 \pm 8 \text{ \AA}$. Upon the addition 150 μM VRL ($N=984$), a reduction in the average height of C-side features was noted, measuring $67 \pm 8 \text{ \AA}$ (Fig 6E, yellow). The number of C-side features of Pgp analyzed before and after adding VCR were 935 and 847, respectively. The introduction of 150 μM VCR decreased the mean height from $69 \pm 9 \text{ \AA}$ (apoPgp) to $68 \pm 8 \text{ \AA}$ (Fig 6E, green).

3.5 DISCUSSION

The clinical application of some of the most pre-clinically potent chemotherapeutic agents is often limited by extreme non-target cytotoxicity. The action of Pgp, which pumps these drugs out of cells, necessitate the use of higher than necessary physiologically relevant dose, resulting in intolerable systemic toxicity. The use of Pgp inhibitors, concurrently with anticancer agents, has been touted but yielded little success (Lai et al., 2020). Moreover, effectively inhibiting the function of Pgp may pose other serious health complications since the protein plays a crucial role in protecting susceptible tissues, such as those of the brain and testis. A better approach to efficient chemotherapy would involve the development of chemotherapeutic agents capable of overcoming Pgp-mediated efflux. A comprehensive understanding of the mechanisms underlying the differential transport of drugs, such as the VAs, is required to address this challenge. In this regard, a model aimed at elucidating the intricate process of Pgp-mediated transport of the VAs is presented in Figure 7.

In silico (Iqbal et al., 2022) and biophysical data (Aanismaa & Seelig, 2007; Romsicki & Sharom, 1999) collectively suggest the existence of at least two distinct binding sites on the protein for the VAs: one characterized by high affinity and the other, low affinity. The high-affinity site is purportedly located closer to the outer leaflet of the membrane, within the conventional central cavity (Iqbal et al., 2022; Nosol et al., 2020). The lower-affinity site is located on the surface of the protein around the protein-lipid interface, closer to the inner leaflet of the membrane (Iqbal et al., 2022). We proposed that at low substrate concentrations, VAs bind to the high-affinity binding sites within the central cavity of the protein, stimulating ATP binding and hydrolysis (Fig. 7). The rate of ATPase activity induced by VBL at relatively lower concentration was approximately 2- and 9-fold higher compared to VRL and VCR, respectively (Fig. 2). Given that other previous

reports indicate a direct interrelationship between the rate of Pgp-mediated ATP hydrolysis and transport for most substrates (Li-Blatter et al., 2012) including VBL (Ambudkar et al., 1997), we infer that among the three drugs tested, VBL is the most effectively transported by Pgp at lower drug concentrations. Applying the same analogy, VCR would be expected to be the least transported. This observation aligns with experimental data showing that the transport rates among the tested VAs are in the order $VBL > VRL > VCR$ (Ferguson & Cass, 1985; Lin et al., 2011; Pierré et al., 1992). Pgp-mediated stimulation of ATPase activity and transport may typically be underpinned by two primary mechanisms: Pgp-ligand binding affinity and ligand-induced conformational changes in the protein. Binding studies employing ligand-induced quenching of tryptophan residues (Fig. 3) revealed that at low substrate concentrations, the affinity of VBL to Pgp is approximately 116% and 225% higher than that of VRL and VCR, respectively. These findings are further reinforced by the identification of potential moieties in VAs engaged in Pgp-VAs interactions. Protons linked to the C₃₀ moiety (Fig. 1 and Fig. 5), which characteristically distinguishes VBL from VCR, displayed notable but varying STDD amplification factors, with VBL exhibiting the highest level of interaction. Furthermore, a previously solved cryo-EM structure of VCR-bound Pgp indicates the possibility of an aldehyde-amide condensation reaction involving C₃₀ moiety on the VAs (Nosol et al., 2020). This condensation reaction may also contribute significantly to the disparities observed in the differential affinity of the VAs to Pgp. Surprisingly, conformational studies (Fig 4) showed that the VAs have no significant impact on Pgp conformation at low substrate concentrations (10 μ M). This finding was unexpected, as most Pgp substrates typically induce notable shifts in the protein's conformation (Pan & Aller, 2018; Zoghbi et al., 2017). However, it is likely the concentration used (10 μ M) might not have been sufficient to saturate the high-affinity binding site and induce observable conformational changes.

An earlier study that employed hydrogen-deuterium exchange mass spectrometry also reported similar observations for VBL (Clouser & Atkins, 2022). Based on our data and previously reported studies (Clouser & Atkins, 2022; Iqbal et al., 2022; Nosol et al., 2020), it appears that at the concentrations tested (10 μ M), differences in ligand-induced conformational changes in the protein may not be the bottle-neck for transport. Instead, the differential binding affinity likely serves as the rate-limiting mechanism governing the transport of VAs by Pgp. Note that these results do not rule out the possibility of ligand-induced changes in the conformational dynamics of individual peptide segments that facilitate the coupling between the drug binding sites and the NBDs. While such changes may not be readily detectable using the techniques employed in this study, they could still be significant enough to stimulate transport. For instance, although the binding of VBL (Crowley et al., 2009, 2010) and VCR (Nosol et al., 2020) to Pgp did not result in significant structural differences in the overall protein structure compared to the drug-free state, alterations in the structure of transmembrane helix 12 (TM12) were observed upon binding (Nosol et al., 2020). These alterations are expected as TM12 plays a pivotal role in mediating communication between the central drug-binding cavity and NBDs (Chai et al., 2022).

Interestingly, at higher drug concentrations, the VAs seem to occupy the low-affinity site, purportedly located at the interface between the protein and the lipid bilayer (Iqbal et al., 2022). At these higher drug concentrations, the ATPase activity induced by the VAs were inhibitory (Fig. 2). Two available theories can explain the observed inhibition of ATPase activity by the VAs. Firstly, numerous studies have linked the traditionally elevated basal ATPase activity of Pgp to the presence of endogenous substances, such as cholesterol, within the lipid bilayer (Garrigues et al., 2002; Tran et al., 2023). Although not transported by Pgp (Al-Shawi et al., 2003), these endogenous lipids may be flip-flopped between the lipid bilayer leaflets (Sharom, 2014;

Thangapandian et al., 2020). A ligand, interacting with the same binding sites as these endogenous substrates but at a slower rate, may appear to inhibit the rate of ATPase activity since the observed Pgp-mediated ATPase activity encompasses both the basal and ligand-induced activation. This line of reasoning is supported by the suggested location of the inhibitory binding sites of the VAs (Iqbal et al., 2022) and the notion that Pgp-mediated ATPase activity depends on a complex interplay between ligands, the membrane environment, and the protein itself (Li-Blatter et al., 2012). The second theory posits that VAs exhibit behaviors akin to certain inhibitors such as tariquidar and verapamil. These inhibitors act as substrates at low concentrations and inhibitors at higher concentrations (Ledwith, Gibbs, et al., 2016; Nosol et al., 2020). The latter theory, among the two proposed, is the most substantiated by the available literature. When the VAs bind to the second site, they likely impede the necessary protein reorientation required for substrate transport, a phenomenon also observed in some Pgp inhibitors (Nosol et al., 2020). However, unlike potent inhibitors, the VAs exhibit relatively lower affinity for the second binding site (Fig. 3), making them unlikely candidates as potent Pgp inhibitors. Studies have revealed that the second binding site is surrounded by multiple transmembrane helices (TM4, TM10, TM9, and TM12) (Nosol et al., 2020). Additionally, the surface of the inhibitory/second binding site features several phenylalanine residues that interact with the bound ligand (Iqbal et al., 2022; Nosol et al., 2020), suggesting that the binding of the VAs at this site may heavily depend on weak hydrophobic interactions. The binding of Pgp inhibitors to the inhibitory binding sites on Pgp appears to restrict TM9 and TM10 (Nosol et al., 2020), the two transmembrane helices known to prevent substrate translocation (Han & Zhang, 1999; J. Song & Melera, 2001). The inhibitory site is also believed to obstruct the access tunnel and vestibule of the protein, preventing the necessary shift when a substrate binds to the central binding cavity (Nosol et al., 2020). The K_i values extracted from the

ATPase kinetic curves for VAs (Fig. 2) suggest that VCR has the highest inhibitory potential at higher substrate concentration, followed by VBL and VRL. In addition, the order of affinity to Pgp at the inhibitory binding site, $K_{D,L}$, is a reverse of the stimulatory binding site (Fig. 3, Table 1). VCR exhibited the highest binding affinity at the inhibitory site, followed by VRL.

Even though there were some differences in the Pgp conformational changes induced by the VAs at 150 μ M, these changes were not substantial. These observations concur with the AFM imaging results, which assessed changes in the distribution of the Pgp protrusion heights within the cytosolic region when exposed to 150 μ M VAs (Fig 6). VBL (Fig 6B) and VRL (Fig 6C) had minimal impact (~ 1 Å) on the average C-side height. VCR (Fig 6D) shifted the distribution the most, though still a modest 1.4 Å difference in the means. This indicates a greater increase in the number of C-side features with heights corresponding to wide open-inward conformations associated with a shorter C-side protrusion. Studies have shown that the activation of Pgp-mediated transport is often dependent on ligand-induced conformational changes in Pgp (Pan & Aller, 2018; Zoghbi et al., 2017). Pgp-mediated transport would be minimal when the protein is in a predominantly wide opened inward conformation (Sigdel et al., 2018). Based on these results, we can infer that, compared to the other two tested drugs, VCR exerts the highest inhibitory influence on Pgp-mediated transport under saturating substrate concentrations. The inhibitory potential is evident from the ability of VCR to shift the protein's conformation, resulting in a 1.33-fold increase in the widely open-inward conformation relative to the apo condition (Fig. 6 & Fig. S1). This is a slightly larger effect compared to that of VBL and VRL, which increased open-inward conformations 1.28-fold and 1.23-fold respectively. The subtle conformational changes detected via the sensitive AFM probe may be associated with the underlying structural mechanism mediating the transport of VAs by Pgp.

Remarkably, when AMPPNP was present, VAs at low concentrations (10 μ M) induced a conformational shift in Pgp similar to that caused by saturating AMPPNP alone (Fig 4), indicating an open-outward conformation. This similarity in conformational changes might be attributed to the low substrate concentration used in comparison to the saturating concentration of AMPPNP. At both saturating drug (150 μ M) and AMPPNP (3.2 mM) concentrations, the VAs induced a shift in Pgp conformation that is intermediate between open-inward and open-outward (Fig 4). However, the conformational shift induced by saturating VCR (Fig. 4H) in the presence of AMPPNP was lesser than those observed for VBL (Fig. 4D) and VRL (Fig. 4F). On the contrary, there was no significant difference observed between the effects of VBL (Fig. 4D) and VRL (Fig. 4F).

There is a demand for the next generation of VAs with increased response rates and manageable toxicity. The development of vinflunine, a third-generation vinca alkaloid with lower toxicity, is promising (Giuseppina et al., 2011; Kruczynski & Hill, 2001; Médioni et al., 2016). Unfortunately, vinflunine is less potent and requires at least a 17-fold higher concentration for a similar effect as VCR (Bachner & De Santis, 2008; El Hayek et al., 2005; Kruczynski et al., 1998). Secondly, the development of new derivatives has been laborious with little success. Identification of functional groups involved in Pgp-VAs interactions may offer new hope for a rational drug design. The STDD NMR revealed that comparable groups on the VAs are involved in Pgp interactions. Peaks from proton linked to C₂₇ on the indoline group of the vinca alkaloids (see Fig 1 and Fig. 5) showed the highest interaction with the protein. In addition, the methyl groups appear to be highly involved in drug-Pgp interactions, as shown by the significant ¹H 1D STDD NMR signals from C₃₀, C₄₁, C₄₂, C₄₂, C₅₀, C₅₆, and C₅₇. Perhaps this explains why substituting a methyl group on VBL (see Fig.1; N₁₁) with an aldehyde (VCR) resulted in disparate Pgp-mediated efflux

rates between the two drugs. VBL showed the highest average STDD amplification factor, which supports the drug-protein affinity data (Fig. 3). Reducing hydrogen bond acceptor (HBA) groups (Li-Blatter et al., 2009; Seelig, 2020; Seelig & Landwojtowicz, 2000) at regions implicated in Pgp-drug recognition revealed by the STDD NMR analysis (Fig. 5) could potentially lead to the development of next-generation VAs capable of evading the transporter. While this approach holds promise for the development of improved derivatives, a limitation lies in the potential loss of function for these drugs. Nonetheless, our understanding of the functional groups responsible for the binding to Pgp, coupled with available SAR studies for the VAs, can guide our search for more effective next-generation vinca alkaloid derivatives

Fig. 7 illustrates a model derived from the summary of our data and existing literature that depicts a probable mechanism for Pgp-mediated transport of VAs. At low substrate concentrations, VAs bind to the stimulatory binding site located within the central cavity of the protein. During this phase, no significant conformational change takes place. However, subtle alterations in TM 12 promote ATP binding at the NBDs, causing the protein to shift into an open-outward conformation, thus facilitating transport (Fig 7A →7B →7C). However, at high substrate concentrations, VAs occupy both the stimulatory and inhibitory binding sites on Pgp. With both binding sites engaged, ATP binding induces an intermediate conformational state between open-inward and open-outward (Fig 7A →7B→7D→7E). This intermediate conformation inhibits Pgp-mediated ATP hydrolysis and transport.

In summary, our findings elucidate a probable structural mechanism governing the Pgp-mediated efflux of VAs. We have also identified the potential functional groups on VAs that could be involved in substrate recognition and binding to Pgp. These findings, coupled with SAR studies

involving VAs and microtubules, could lay the foundation for rational drug design aimed at the next-generation VAs that are potent and show less systemic toxicity.

3.6 FIGURES

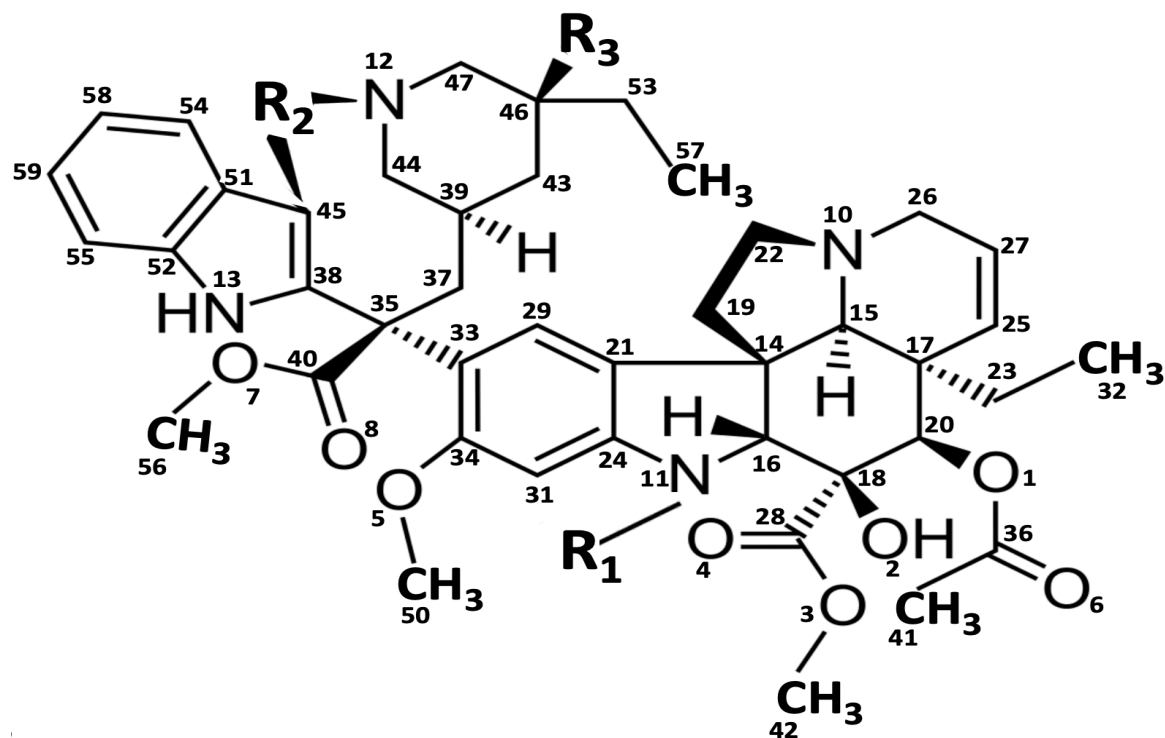


Figure 3.1 The basic structure of the Vinca Alkaloids (VAs)

	R1	R2	R3
VBL	³⁰ -CH ₃	⁴⁸ ⁴⁹ -CH ₂ CH ₂	⁹ -OH
VRL	³⁰ -CH ₃	⁴⁸ -CH ₂ -	—
VCR	³⁰ -C=O	⁴⁸ ⁴⁹ -CH ₂ CH ₂	⁹ -OH

Figure 3.1 The basic structure of the Vinca Alkaloids (VAs). The table shows the figure legend representing Vinblastine (VBL), Vinorelbine (VRL) and Vincristine (VCR).

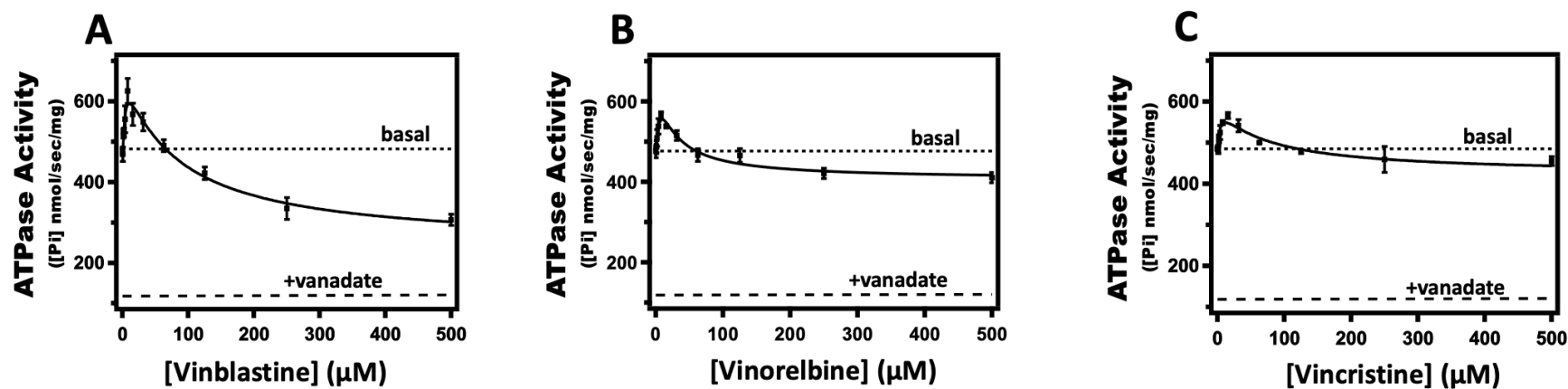


Figure 3.2 The effect of the vinca alkaloids on Pgp-mediated ATP hydrolysis

Figure 3.2 The effect of the vinca alkaloids on Pgp-mediated ATP hydrolysis. Ligand induced Pgp-coupled ATPase activity in the presence of increasing concentrations of (A) VBL (closed squares), (B) VRL (closed squares), and (C) VCR (closed squares). For comparison, the basal and vanadate inhibited ATPase activity are shown as dotted line and dashed-line, respectively in each panel. Each data point represent the average of at least 3 independent experiment and the error bars shown represent the standard deviation.

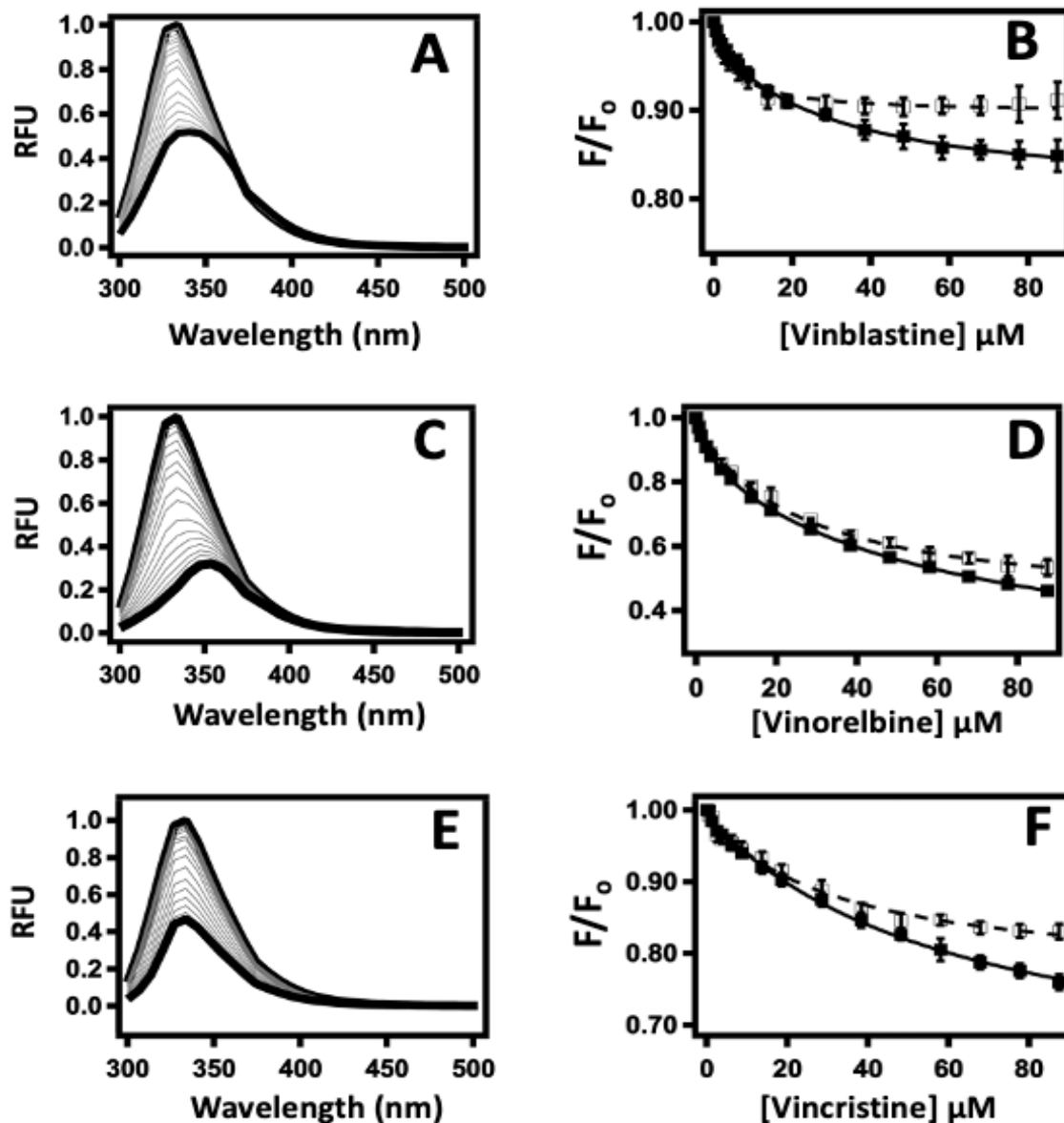


Figure 3.3 The binding affinity of vinca alkaloids to Pgp determined by the quenching intrinsic tryptophan fluorescence by ligands

Figure 3.3: The binding affinity of vinca alkaloids to Pgp determined by the quenching intrinsic tryptophan fluorescence by ligands. A normalized Fluorescence emission decay spectra of Pgp following excitation at 280 nm in the presence of increasing (A) VBL, (C) VRL, and (E) VCR concentrations. The upper and lower thick lines represent the absence (0 μM) and the presence of saturating concentrations (150 μM) of the VAs. The fluorescence spectra at the intermediate concentrations are shown in gray lines between the thick lines. The corrected fluorescence

emission amplitude at 330 nm were plotted as a function of the concentration of (B) VBL, (D) VRL, and (F) VCR in the absence (closed square) and presence (opened square) of saturating (3.2 mM) AMPPNP. Each data point and error bar represent the average and standard deviation of at least three independent experiments.

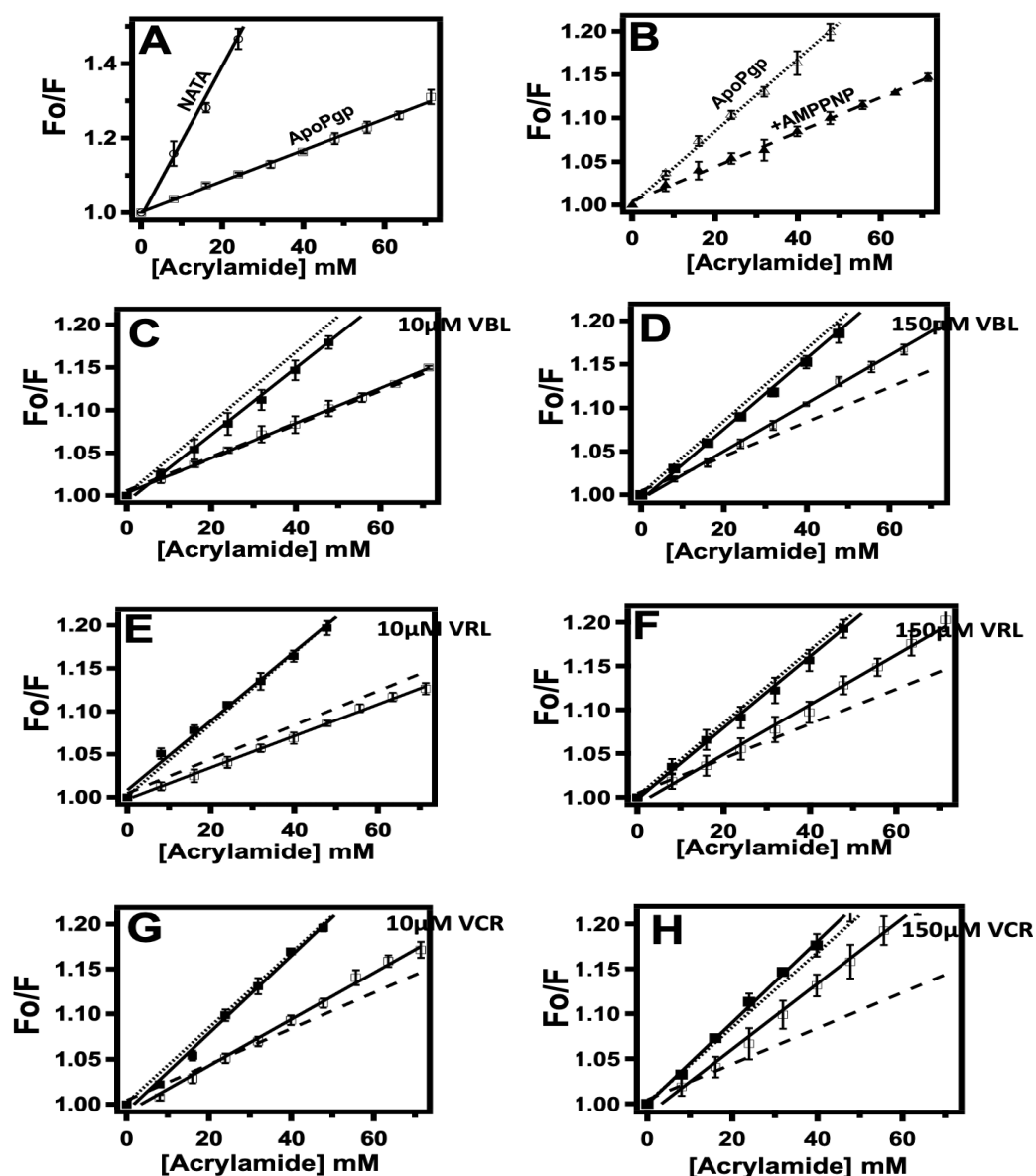


Figure 3.4 Vinca alkaloid-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence

Figure 3.4 Vinca alkaloid-induced conformational changes in Pgp probed by acrylamide quenching of protein fluorescence. (A) Stern-Volmer plot for NATA (open circles) and ApoPgp (open squares). (B) Stern-Volmer plot for Pgp fluorescence in the absence (open triangle, dotted line) and presence of 3.2 mM AMPPNP (closed triangle, dashed line). Panels (C) and (D) depict the fitted Stern-Volmer plots of Pgp induced by 10 μM and 150 μM of VBL, both in the absence (closed square) and presence (open square) of 3.2 mM AMPPNP. (E) 10 μM and (F) 150 μM

VRL-induced Stern-Volmer plots of Pgp fluorescence in the absence (closed square) and presence (open square) of 3.2 mM AMPPNP. For comparison, Stern-Volmer plots of ApoPgp without (dotted lines) and with AMPPNP (dashed lines) are shown in panels C-H. Data points and error bars represent the mean and standard deviation, respectively, from a minimum of three independent experiments.

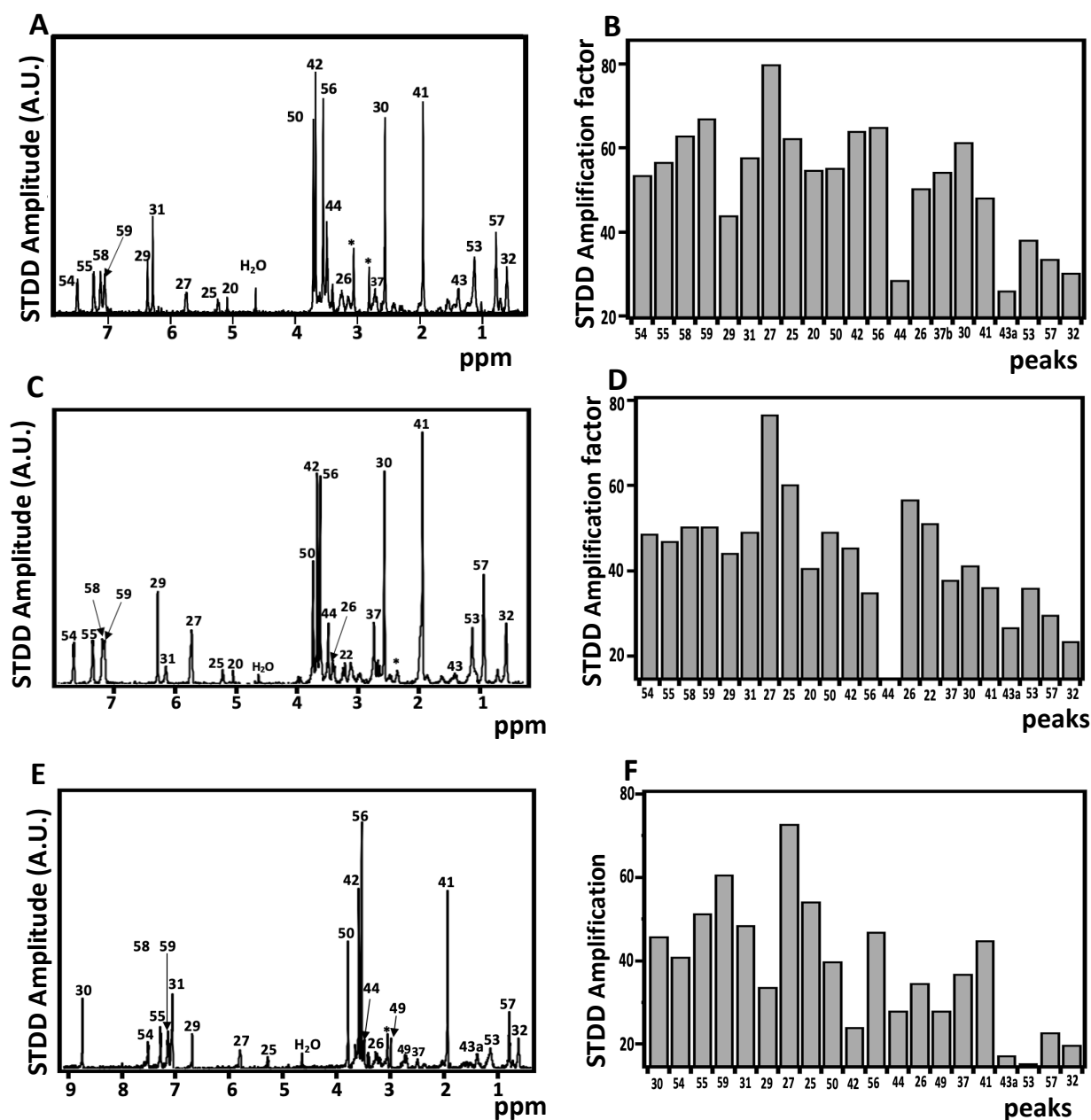


Figure 3 5 The functional groups of vinca alkaloids involved in its interactions with Pgp investigated by STDD NMR

Figure 5: The functional groups of vinca alkaloids involved in its interactions with Pgp investigated by STDD NMR. (A) (C), and (E) represent the ^1H 1D STDD NMR spectrum of VBL, VCR, and VRL, respectively. A category plot of STDD amplification factors deduced for (B) VBL, (D) VRL, and (F) VCR, respectively, were estimated from the ^1H 1D STDD NMR spectrum. The peak labels in each panel correspond to protons in Figure 1. Sub-label ‘a’ and ‘b’ represents axial and equatorial protons, respectively. The * represent unidentified peaks

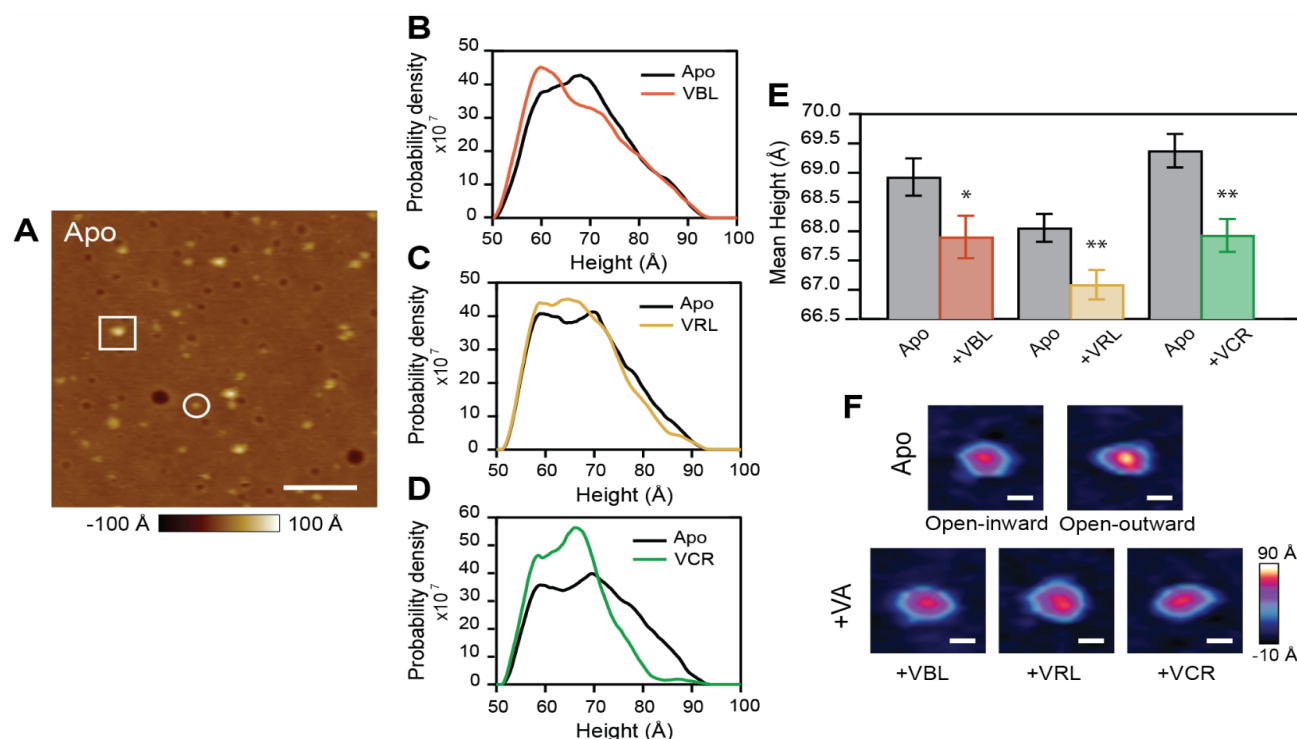


Figure 3.6 The effect of vinca alkaloids on the height distributions of Pgp at the cytosolic domain (C-domain) analyzed deduced AFM imaging

Figure 3.6: The effect of vinca alkaloids on the height distributions of Pgp at the cytosolic domain (C-domain) analyzed deduced AFM imaging. Smooth histograms illustrating population shifts from apo (black) and in the presence of VBL (A; red), VRL (B; orange), and VCR (D; green). The probability density shown on the y-axis of panels A, B, and C, was determined by integrating histogram area using SI units. (D) The heights of the populations are compared side-by-side to show relative shifts of drug-induced conformations from apoPgp; error bars are the standard deviation of the individual Gaussian for that population. (E) The probability density difference (Δ PD) relative to the apo state indicates how the fraction of features within the tallest populations shift in the presence of each vinca alkaloid.

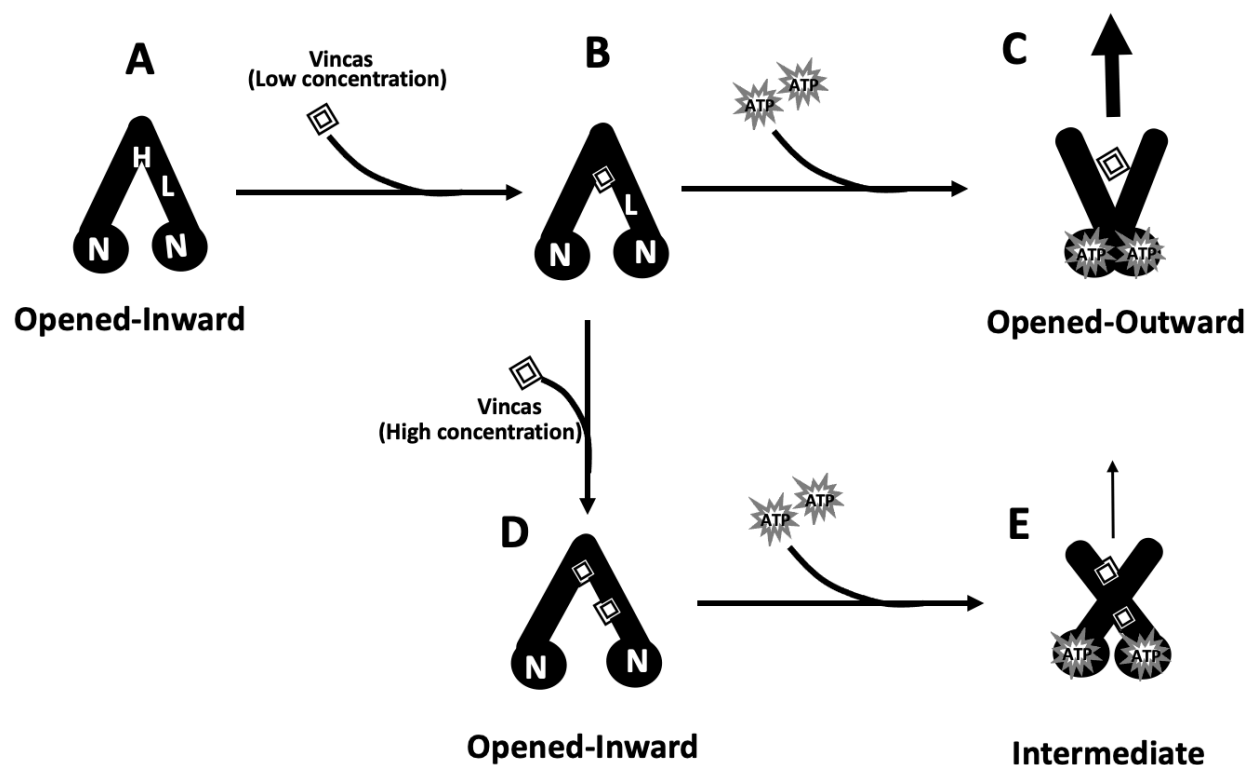


Figure 3.7 Model illustrating VA-induced activation of Pgp-mediated ATP hydrolysis and transport

Figure: 3.7 Model illustrating VA-induced activation of Pgp-mediated ATP hydrolysis and transport. The figure illustrates the cartoons of Pgp in the open-inward (A, B, D), opened outward (C) and intermediate (E) conformations. Pgp is represented in solid black, with the black circular region labeled 'N' representing the nucleotide-binding domain (NBD). Molecules of vinca alkaloids are shown as diamonds. H and L depict the proposed high-and low-affinity binding sites, respectively, of the vinca alkaloids on Pgp. The size of the vertical arrows reflects the degree of Pgp-mediated ATP hydrolysis and transport of the vinca alkaloids.

Table 3.1 Kinetic parameters and the corresponding standard deviations deduced by fitting the rate of Pgp-coupled ATPase activity curves over a range of vinca alkaloid concentrations in Figure 2

Substrate	Basal Activity (nmol/min/mg)	K_m (μM)	K_i (μM)	V_{max} (nmol/min/mg)
VBL	483 ± 41	1.78 ± 2.34	24.17 ± 6.12	601 ± 30
VRL	478 ± 11	5.12 ± 3.87	8.79 ± 4.76	590 ± 21
VCR	471 ± 15	17.04 ± 6.50	7.40 ± 3.26	558 ± 33

Table 3.2. Kinetic parameters determined by fitting Pgp-VAs binding affinity curves from Figure 3.

Vinca Alkaloids (VAs)	$K_{\text{sv,H}} (\mu\text{M}^{-1})$	$K_{\text{D,H}} (\mu\text{M})$	$K_{\text{sv,L}} (\mu\text{M}^{-1})$	$K_{\text{D,L}} (\mu\text{M})$
VBL	0.849 ± 0.060	1.18 ± 0.07	0.015 ± 0.004	67.67 ± 5.08
VBL (+AMPPNP)	0.052 ± 0.006	19.26 ± 2.25	-	-
VRL	0.393 ± 0.087	2.55 ± 0.16	0.032 ± 0.010	30.96 ± 5.16
VRL (+AMPPNP)	0.040 ± 0.019	25.00 ± 2.49	-	-
VCR	0.260 ± 0.028	3.84 ± 0.61	0.040 ± 0.003	24.89 ± 5.64
VCR (+AMPPNP)	0.104 ± 0.038	9.58 ± 3.51	-	-

3.7 SUPPLEMENTARY INFORMATION

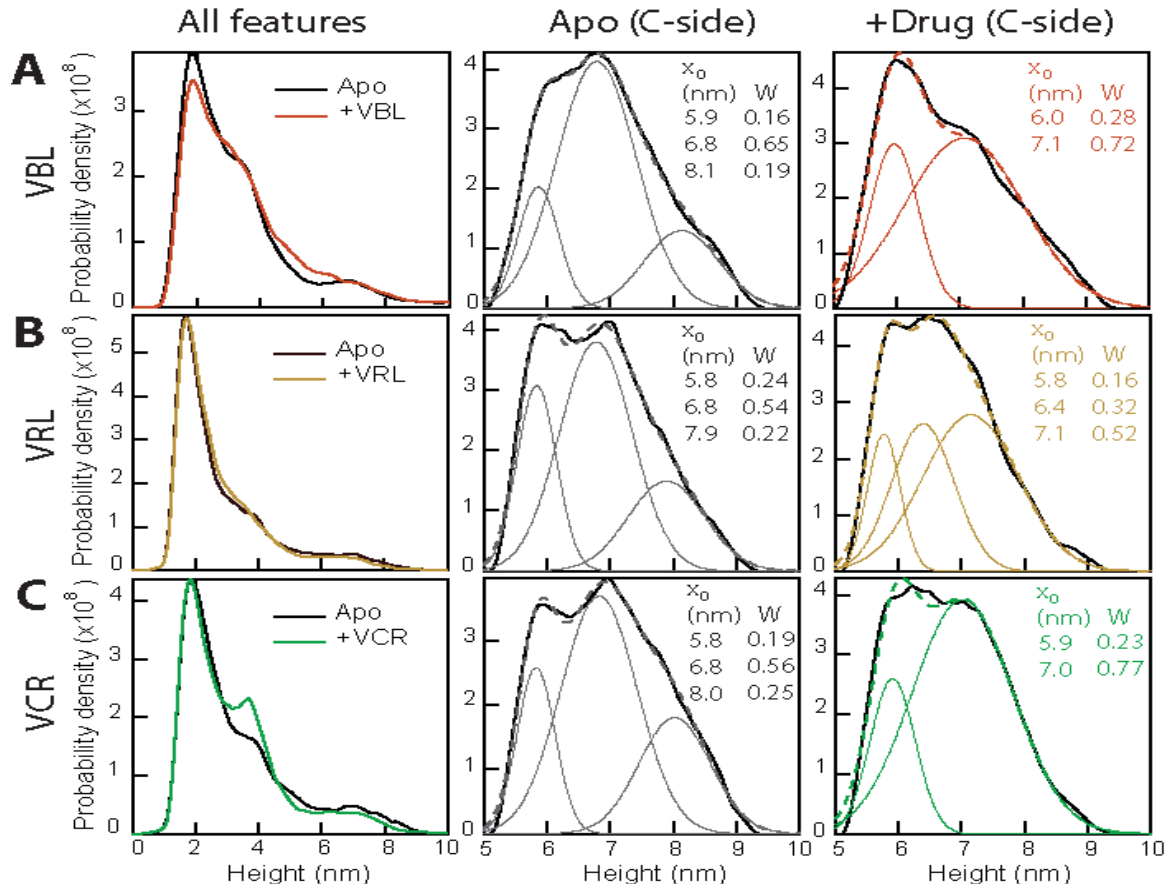


Figure S3.1 Analyses of the height distribution of Pgp in the lipid bilayer

Supplemental Figure 3.1. Analyses of the height distribution of Pgp in the lipid bilayer. The histograms of the height distribution of Pgp in the absence and presence of (A) VBL, (B) VRL, and (C) VCR. The left panels show histograms of all the features analyzed. The C-side apo data (middle panels) and Pgp in the presence of the vinca alkaloids (right panels) were fit using Bayesian information criterion (BIC) to determine the appropriate number of Gaussian distributions. Individual Gaussian distributions (solid-colored lines) were summed together to form the model fit (dashed lines). Raw data are shown in solid black line. In all conditions, the apo required 3 Gaussians, while the Pgp in the presence of the vinca alkaloid conditions varied. The peak positions and weights are presented to the left of the graphs.

CHAPTER 4

DRUG-INDUCED CONFORMATIONAL DYNAMICS OF P-GLYCOPROTEIN

UNDERLIES THE TRANSPORT OF CAMPTOTHECIN ANALOGS⁴

⁴ Mensah, GAK., Bartlett, MG, Roberts, AG. To be submitted to *Lipids in Health and Disease Journal*

4.1 ABSTRACT

Cholesterol, a prominent component of the lipid bilayer, has been implicated in cancer progression and chemotherapy outcomes. This study explores the multi-faceted interactions between Pgp and derivatives of cholesterol, shedding light on both direct and indirect mechanisms involved. Directly, Pgp interacts with cholesterol derivatives and facilitates the trans-bilayer motion of cholesterol via flip-flop. Sterols like 7-ketocholesterol (7KC) exhibit enhanced basal Pgp activity due to the presence of the extra polar head residue that enables robust interaction with the Pgp. Conversely, cholesterol (CHL), with its greater hydrophobicity, promotes the partitioning of hydrophobic ligands into the interleaflet space where the central binding cavity of Pgp resides. The enhanced partitioning facilitates the binding and translocation of substrates by Pgp. In addition, the distinct orientation of CHL in the membrane, compared to other oxidized CHL, promotes membrane rigidity, and favors a transport-conducive orientation that reduces the conformational barrier in the protein. The reduction of the structural barrier facilitates ATP binding and promotes the conformational dynamism required for successful Pgp-mediated translocation of substrates.

4.2 INTRODUCTION

P-glycoprotein (Pgp) is a highly conserved member of the B-class ATP-binding cassette (ABC) superfamily present in various eukaryotic and prokaryotic organisms (El-Awady et al., 2017). Discovered in 1976 (Juliano & Ling, 1976), Pgp is an ATP-dependent transporter known for its ability to actively pump a diverse array of molecules out of cells, even against concentration gradients. Physiologically, the transporter protects cells by pumping out potentially harmful exogenous and endogenous molecules out of cells (Liang et al., 2015). Unsurprisingly, the protein is highly expressed in the apical membrane of many normal secretory cells (renal tubular cells, bile canaliculi, intestinal epithelial cells) and physiological blood-tissues barriers (blood-brain, blood-placenta, and blood-testis) (Amin, 2013; Y. Li et al., 2010). Tumor cells tend to overexpress Pgp, which can confer on them multi-drug resistance (MDR) phenotypes (Zhou, 2008). Unfortunately, among the substrates of Pgp are a broad range of essential therapeutic agents, including several anti-cancer drugs (Research, 2021). By transporting chemotherapeutic agents out of cells, Pgp potentially prevents the accumulation of these life-saving drugs to therapeutic levels, thereby limiting their cytotoxic or apoptotic effect (Callaghan et al., 2014). The development of MDR in tumor cells has been implicated in about 90% of deaths in cancer patients on chemotherapy (Bukowski et al., 2020).

Pgp being a transmembrane protein, lipids are believed to play a vital role in the function of the protein (Renard & Byrne, 2021; Sharom, 2014; Thangapandian et al., 2020; Tran et al., 2023). Lipids are believed to provide the structural support for Pgp within the cell membrane and influence its localization within lipid rafts where Pgp is predominantly localized. Lipids play a role in the interactions between Pgp and its substrates, but the details of these interactions remain murky. These factors collectively contribute to the ability of the protein to pump drugs and toxins

out of cells. Studies have shown that changes in lipid composition can create a variety of domains with altered fluidity and rigidity of the membrane (Casares et al., 2019). These changes in lipid composition can impact Pgp's ability to transport substrates (Romsicki & Sharom, 1999). In fact, available data suggest that Pgp requires a lipid layer to activate and subsequently transport its substrates (Loo & Clarke, 2016). The membranes of eukaryotes are made of three main types of lipids: phospholipids, cholesterol, and glycolipids (Alberts et al., 2002).

Cholesterol is a polycyclic amphipathic molecule that forms a vital component of the eukaryotic cell membrane. Cholesterol makes up 20-30 percent of the mammalian membrane (Elkins et al., 2018), highlighting its importance to the proper membrane functioning. In the plasma membrane, this lipid helps to maintain integrity, reduce fluidity, and improve the membrane's mechanical stability (Yang et al., 2016). The relationship between cholesterol and membrane protein does not only vary from protein to protein; it is complex and poorly understood (Elkins et al., 2018). It is believed that the structural characteristics of cholesterol enable it to potentially modulate protein-membrane interactions directly with membrane proteins or via non-specific effects on the physical properties of the membrane as a whole (Fantini & Barrantes, 2013). Previous studies indicate that Pgp predominantly localizes in cholesterol-rich microdomains of eucaryotic plasma membranes (Sharom, 2014), a phenomenon yet to be fully resolved. Other reports have revealed that cholesterol may play a role in the correct protein folding and provide stability for the protein, as observed in other membrane-associated proteins such as (Sharpe et al., 2019). Studies have shown that changes in the lipid bilayer's cholesterol composition influence cell-specific and substrate-dependent effects on the transport activity of Pgp (Luker et al., 2000). Recent reports also suggest that cholesterol modulates the basal and drug-stimulated ATP hydrolysis and the drug-binding and transport potential of Pgp (Fenyvesi et al., 2008;

Thangapandian et al., 2020). Fluorescently labeled verapamil in LY335979 cells overexpressing Pgp revealed that increasing cholesterol concentration increases the activity of the Pgp (Troost et al., 2004). Other *in vitro* studies revealed that membrane cholesterol linearly modulates Pgp-mediated ATPase activity (Gayet et al., 2005; Riou et al., n.d.). Unfortunately, little is known about the complex Pgp-cholesterol interplay and the factors mediating this relationship. Even though an earlier study inferred that the enhanced activity of Pgp in the cholesterol-rich domain of the membrane was due to the preferential accumulation of substrates in these regions (Subramanian, Condic-Jurkic, et al., 2016), this theory could not account for the increased basal ATPase activity of the Pgp in the absence of substrates. In this study, we investigated the potential structural and biochemical mechanism underlying the effect of cholesterol and some of its derivatives on the activity of Pgp in a biosynthetic system. The rate of drug-induced Pgp-mediated ATP hydrolyses was investigated to identify the modulating effect of cholesterol on the basal and drug-stimulated Pgp-mediated ATP hydrolysis. Cholesterol-induced Pgp conformational changes were determined directly and indirectly by acrylamide quenching of Pgp intrinsic tryptophan fluorescence spectroscopy.

4.3 EXPERIMENTAL PROCEDURE

4.3.1. Chemical Reagents

Disodium ATP, cholesterol, and Tris-HCl were purchased from Amresco (Solon, OH). Adenosine 5'-(β,γ -imido)triphosphate lithium salt (AMPPNP), acrylamide, N-acetyl-L-tryptophanamide (NATA), ammonium chloride (NH_4Cl), and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (Milwaukee, WI). Nitrilotriacetic acid (NTA) and diethyl aminoethyl (DEAE) cellulose resin were procured from Thermo Fisher Scientific. Ethylene glycol tetraacetic acid (EGTA) and imidazole were acquired from Alfa Aesar (Tewksbury, MA). Dithiothreitol (DTT) was obtained from Gold Biotechnology (Olivette, MO). Sodium orthovanadate (Na_3VO_4) was purchased from Enzo Life Sciences (Farmingdale, NY). The detergent, n-dodecyl- β -D-maltoside (DDM), was acquired from EMD Millipore Corporation (San Diego, CA). Escherichia (E.) Coli total lipid extract powder, 7-ketocholesterol, and 25-hydroxy-cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All other reagents and chemicals, except otherwise stated, but used in this study were purchased from Thermo-Fischer Scientific.

4.3.2 Methods

4.3.2.1 Expression and Purification of P-glycoprotein

Genetically engineered strains of *Pichia pastoris* modified to overexpress His-tagged wild-type mouse Pgp (MDR3) were cultured in glycerol media. The yeast culture was further induced with methanol for efficient protein production at the Bioexpression and Fermentation Facility (BFF), University of Georgia, Athens, GA, as previously described (Lerner-Marmarosh et al., 1999; Urbatsch et al., 1998). At least six alternative liquid-nitrogen freezing, blending, and thawing were carried out to lysed the harvested cells (Dunn & Wobbe, 2001). Finally, a two-step

approach was used to purify the protein as previously described (Bai et al., 2011; Lerner-Marmarosh et al., 1999) but with few modifications. The first step employed an affinity-chromatography technique that allowed the his-tagged protein to bind to the nickel-nitrilotriacetic acid (Ni-NTA) (ThermoFisher Scientific, Waltham, MA) resin. Next, ion-exchange chromatography with diethylaminoethyl cellulose (DEAE) resin (ThermoFisher Scientific) was utilized, as previously described (Bai et al., 2011; Lerner-Marmarosh et al., 1999). The purified proteins were solubilized in DMM and concentrated using Amicon Ultra-15 100 kDa cut-off filters (EMD Millipore, Billerica, MA). The average yield for the purified protein was determined with a Nanodrop (DeNovix, PHL). The concentration of the purified protein was determined using a molar absorption coefficient of 1.28mlmg^{-1} (Bai et al., 2011). An SDS/PAGE analysis of the purified protein was carried out as previously described (Ledwitch, Barnes, et al., 2016; Lerner-Marmarosh et al., 1999) and showed >95% purity. The detergent-solubilized proteins were stored at -80°C in aliquots.

4.3.2.2 Reconstitution of Pgp into liposomes

The liposomes were made from Avanti E. coli total lipid extract (80% w/v) and cholesterol (20% w/v) mixed in chloroform to a final volume and concentration of $10\mu\text{L}$ and 10mgml^{-1} , respectively. The chloroform in the organic mixture was evaporated with Buchi Rotavapor, Model R-114 (Buchi, DE), to form a dry film. The dried film generated was re-suspended with 10mL of rehydration buffer (0.1 mM EGTA and 50 mM Tris-HCl solution with a pH of 7.4). Liquid nitrogen and a water bath set at 67°C were used to carry out at least ten freeze-thaw cycles to enhance the re-suspension of the dried film in the rehydration buffer and form liposomes of varying sizes. A filter extrusion procedure described previously (Wilt et al., 2017) was used to form unilamellar liposomes with an average size of 250 nm. The liposomes were extruded 11 times

through a 400 nm cut-off filter (EMD Millipore) using the LIPEX extruder (Northern Lipids, Burnaby, British Columbia, Canada). A Dynamic Light Scattering (DLS) analysis showed homogeneously sized extruded liposomes with an average size of 250 nm. To reconstitute the purified P-gp into liposomes, about 50 μ L of Pgp solubilized in the detergent was dialyzed against HEPES buffer (20 mM HEPES, 100 mM Sodium Chloride, 5 mM Magnesium Chloride, 2 mM DPP, pH 7.4) for 2 hours to remove residual detergents. Pgp was mixed with 4mgml⁻¹ of the previously extruded liposomes and incubated on ice for 1 hour to promote the integration of the protein into the liposomes. Following incubation, another dialysis was carried out on a HEPES buffer for 2 hours to remove excess detergents and further enhance Pgp integration into the liposomes to form proteo-liposomes. Bio-Rad DC Protein assay was used to determine the concentration of the proteo-liposomes as previously done (Ledwitch, Gibbs, et al., 2016), and aliquots were stored in HEPES buffer, pH 7.4, at -80°C.

4.3.2.3 Pgp-Mediated ATPase Activity Assay

The Chifflet method estimates the activity of Pgp as a function of the concentration of inorganic phosphate, Pi, produced during Pgp-mediated ATP hydrolysis. The inorganic phosphates produced form complexes with molybdenum, which characteristically has a high absorbance at 850nm. Pgp-mediated ATP hydrolyses in the presence of a range of cholesterol, 25-hydroxy-cholesterol, and 7-keto-cholesterol concentrations were measured using Chifflet's procedure as previously described (Chifflet et al., 1988) with slight modifications. The absorbance of the samples was measured at 850 nm on a 96-well plate using a FlexStation (III) spectrometer (Molecular Device, Sunnyvale, CA). The final concentration of the reconstituted P-gp used was 50nM in Chifflet buffer (150mM NH₄Cl, 5mM MgSO₄, 0.02% wt/vol NaN₃, 50mM Tris-HCl, pH=7.4). Due to the limitations of linear regression fitting curves, a non-linear regression (a modified Michaelis-

Menten equation) was used to fit the ATPase activity kinetic curves (Segel, 1993). The Pgp-mediated ATPase activity curve generated, if monophasic, is fitted into the equation shown below (Eqn1)

$$V = \frac{V_{\max}[S]}{K_m + [S]} + V_{\text{basal}} \quad (1)$$

Where **V** is the ATP hydrolysis rate, $V_{\max}$ is the maximum ATP hydrolysis at saturating drug concentrations, [S] is the substrate concentration, **K_m** is the Michaelis-Menten constant, **K_i** is the inhibitory constant and **V_{basal}** is the basal ATPase activity. ATP hydrolysis kinetic curves believed to be biphasic are fitted into the equation below (Eqn2)

$$V = \frac{V_{\max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} + V_{\text{basal}} \quad (2)$$

An advanced software, Igor 6.2 pro (Wavemetrics, Tigard, Oklahoma, USA), was used for analysis.

4.3.2.4 Drug-induced Pgp Conformational Changes Probed by Acrylamide Quenching

Pgp is a highly dynamic protein with a wide range of conformational plasticity. (Subramanian, Condic-Jurkic, et al., 2016). The dynamic characteristics of the protein make it an effective efflux pump. Acrylamide is a small polar molecule capable of interacting and reducing the fluorescence of tryptophan residues in proteins (Lakowicz, 2011; R. Liu et al., 2000; Strambini & Gonnelli, 2010). Proteins may undergo conformational changes as they interact with ligands (Cioni & Strambini, 1998). These conformational changes could either occlude or expose the tryptophan residues in the protein, leading to changes in the solvent-accessible tryptophan residues (Russell & Sharom, 2006; Sharom et al., 2003; Sonveaux et al., 1999). Since acrylamide cannot readily

penetrate the hydrophobic interior of the protein, changes in the protein's fluorescence can help predict ligand-induced changes in the tertiary structure of the protein (Eckford & Sharom, 2008; R. Liu et al., 2000; Zoghbi et al., 2017). In this study, the intensity of 1 μ M Pgp reconstituted into liposomes was measured in Chifflet buffer and 2 mM DTT at 330 nm following excitations at 280 nm. The fluorescence intensities were corrected with [Eqn3](#), and a Stern-Volmer plot of $F_{o(corrected)}/F_{(corrected)}$ versus the acrylamide concentration was generated.

$$F_{corrected} = (F - B)10^{\frac{(\epsilon_{exbex} + \epsilon_{embem})[Q]}{2}} \quad (3)$$

The slope of the Stern-Volmer curve, a function of the degree of quenching, is related to the K_{sv} value by [Eqn4](#) below (Lakowicz, 2011):

$$\frac{F_{o(corrected)}}{F_{(corrected)}} = 1 + K_{sv}[Q] \quad (4)$$

4.4 RESULTS

4.4.1 ATPase Activity

Pgp is an energy-dependent efflux pump that requires hydrolysis of at least one ATP molecule per catalytic cycle (Sauna & Ambudkar, 2007; Seelig, 2020; Sharom, 1997). It is believed that the hydrolysis of ATP provides the minimum energy required to reset the conformation of the protein after transport (Patzlaff et al., 2003; Sauna et al., 2002). Pgp-mediated ATPase activity assays have been used previously to identify Pgp substrates/inhibitors (Litman et al., 1997; Seelig, 2020) and to measure effective Pgp-mediated transport of substrates (Ambudkar et al., 1997).

4.4.1.1 The effect of cholesterol and its derivatives on Pgp-Mediated ATP hydrolysis basal activity.

The rate of ATP hydrolysis was used to determine the potential influence of cholesterol on the Pgp-mediated ATPase activity. Figure 2 shows the effect of increasing cholesterol concentrations on the basal activity of Pgp. Panels 2A, 2B, and 2C (closed squares and thick lines) show the kinetic curves generated from the Pgp-coupled ATPase activity in the presence of varying concentrations of CHL, 7-KC, and 25-HC, respectively. Each panel in Figure 2 also shows the basal ATPase activity (dotted lines) and the presence of 200 μ M orthovanadate (short dashes). The basal activity of Pgp-coupled ATPase activity was 432 ± 9.2 nmolmin⁻¹mg⁻¹, 450 ± 4.4 nmolmin⁻¹mg⁻¹, and 447 ± 2.3 nmolmin⁻¹mg⁻¹ for CHL, 7-KC, and 25-HC, respectively, which agrees with previously reported studies (Borgnia et al., 1996; Ritchie et al., 2009; Wilt et al., 2017). The kinetic parameters, K_m and V_{max} , were determined by fitting the Pgp-coupled ATP hydrolysis kinetic curves into a modified Michaelis-Menten equation ([Eqn1](#)). The K_m and V_{max} deduced for CHL were 20.16 ± 11.24 nmolmin⁻¹mg⁻¹ and 542 ± 16 μ M, respectively. The estimated K_m and V_{max} for 7-KC were 56.70 ± 18.8 nmolmin⁻¹mg⁻¹ and 591 ± 4.4 μ M, respectively. For 25-HC, the K_m and

V_{max} were $53.40 \pm 9.2 \text{ nmolmin}^{-1}\text{mg}^{-1}$ and $604 \pm 9.92 \text{ }\mu\text{M}$, respectively. These results imply that the mechanisms underlying the interaction between Pgp and CHL and its derivatives also stimulate ATP hydrolysis.

4.4.1.2 The influence of cholesterol and its derivatives on verapamil-stimulated Pgp-mediated ATP hydrolysis.

Illustrated in Figure 3 is the effect of varying cholesterol composition on the stimulatory effect of verapamil-induced Pgp-coupled ATP hydrolysis. Panels 3A and 3B show the modulating effect of CHL (blue strip) and 7KC (blue strip) on ligand-induced ATPase activity of Pgp in the presence of $30 \text{ }\mu\text{M}$ of verapamil. Each panel in red shows the inhibitory effect of $200 \text{ }\mu\text{M}$ orthovanadate, a phosphatase inhibitor (Broccatelli et al., 2011; Urbatsch et al., 1995). The rate of Pgp-mediated ATP hydrolysis observed for the increasing percentage of CHL and 7KC in liposomes used for the reconstitution of Pgp in the presence of $100 \text{ }\mu\text{M}$ verapamil and $200 \text{ }\mu\text{M}$ orthovanadate were adjusted for concentration of the protein and incubation time. Irrespective of the type and amount of CHLs present in the liposome, $200 \text{ }\mu\text{M}$ of vanadate successfully inhibited Pgp ATPase activity by at least 85%, consistent with previously reported study (Loo et al., 2006). At 0%, 5%, 10%, 15%, and 20% of CHL in liposomes used to reconstitute the protein, the level of Pgp-mediated ATP hydrolyses were $322 \pm 41 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1344 \pm 58 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1564 \pm 68 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1807 \pm 74 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $2158 \pm 49 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively (Fig. 3, Table 1). Beyond 20% composition of CHL in liposomes, the increasing CHL levels result in decreasing ATP hydrolysis rate. The estimated rate of verapamil-induced ATPase activity for Pgp reconstituted into liposomes containing 30%, 40%, and 50% CHL were $2055 \pm 41 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1650 \pm 32 \text{ nmolmin}^{-1}\text{mg}^{-1}$, and $1589 \pm 36 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively. For 7KC, ATPase activity observed for Pgp reconstituted into liposomes made of 0%, 5%, 10%, 15%, 20%, 30%,

40%, and 50% were $274 \pm 61 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $894 \pm 35 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $854 \pm 29 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $990 \pm 63 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1801 \pm 63 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1652 \pm 22 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1609 \pm 48 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1508 \pm 65 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively. 25HC showed a similar pattern but a different degree of influence on verapamil-stimulated ATPase activity. The rate of ATPase activity observed for 0%, 5%, 10%, 15%, 20%, 30%, 40%, and 50% of 25KC liposome composition were $251 \pm 12 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $667 \pm 19 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $835 \pm 31 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $934 \pm 22 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1253 \pm 17 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1436 \pm 25 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1569 \pm 24 \text{ nmolmin}^{-1}\text{mg}^{-1}$, $1564 \pm 42 \text{ nmolmin}^{-1}\text{mg}^{-1}$, respectively.

4.4.2. Probing Cholesterol-Induced Pgp Conformational Changes Probed by Acrylamide quenching of protein florescence.

Pgp is structurally highly dynamic, a characteristic that is influenced by its interactions with ligands. The accessibility of tryptophan residues in the protein by the bulk solvent may change as the structural conformation of the protein is altered (Zoghbi et al., 2017). Tryptophan residues in a protein absorb well at 295 nm and emit at 330 nm wavelength. The amplitude of protein fluorescence was monitored at 330 nm and adjusted for inner filter effects using [Eqn3](#). Acrylamide, a small polar collisional quencher of tryptophan fluorescence, has been used in previous studies to indirectly determine ligand-induced changes in the tertiary orientations of the proteins, including Pgp (Akbar et al., 2016; Gibbs et al., 2018; Jazaj et al., 2019). Pgp exhibits the highest degree of acrylamide quenching when the protein is in an open-inward conformation and the tryptophan residues are readily accessible by the bulk solvent. (R. Liu et al., 2000; Moller & Denicola, 2002). On the contrary, the tryptophan residues become relatively secluded, resulting in a minimized degree of quenching when Pgp is in the opened-outward conformation (R. Liu et al., 2000; Moller & Denicola, 2002; Wilt et al., 2017). Figure 4 shows the Stern-Volmer plots and the corresponding

K_{sv} values of the fluorescence quenching of apoPgp reconstituted into liposomes by acrylamide. Shown in panels 4A and 4B are the Stern-Volmer plot of NATA (dashes), Pgp reconstituted into 0% (solid line), and 50% (dotted line) of CHL composition in liposomes, in the presence and absence of saturating (3.2mM) AMPPNP, respectively. The K_{sv} values deduced by fitting the Stern-Volmer plot in the presence and absence of AMPPNP into [eqn4](#) are shown in panels 4C and 4D, respectively. The K_{sv} values estimate the degree of quenching and are equivalent to the slopes from the fitted kinetic curves. Since acrylamide is unlikely to permeate the hydrophobic interior of neither the protein nor the lipid bilayer (data not shown), a significant K_{sv} value suggests greater accessibility of the tryptophan residues to the bulk solvent. The Stern-Volmer curves shown in this study were linear, indicating acrylamide neither binds directly nor affects the conformation of the protein (Lakowicz, 2011). The K_{sv} value estimated for NATA was $15.43 \pm 0.37 \text{ M}^{-1}$, consistent with previously reported values (Ledwitch, Barnes, et al., 2016; Patra et al., 2015; Strambini & Gonnelli, 2010). The Stern-Volmer plot of NATA serves as a positive control, and an indicator of the maximum degree of quenching had all the 11 tryptophan residues in Pgp been accessible to the bulk solvent (de Foresta et al., 2010). Only 3 of the 11 tryptophan residues in Pgp are believed to be completely solvent-accessible (Swartz et al., 2013, 2020). The K_{sv} value of apoPgp reconstituted in liposomes with increasing CHL percentage composition is shown in Table 1. These results highlight the role membrane CHL has on the structural orientation of Pgp. As the percentage of cholesterol in proteoliposomes increases, the availability of the tryptophan residues to the bulk solvent decreases, leading to a decreasing rate of quenching of the protein fluorescence by acrylamide. Figure 5 shows Stern-Volmer plots of Pgp reconstituted into liposomes with different compositions of 7KC. In Panel 4A and 4B, Stern-Volmer plots are depicted for NATA, Pgp reconstituted in the absence of 7KC (0%), and Pgp reconstituted in the presence of 50% 7KC.

These plots are shown both in the absence and presence of AMPPNP. The Stern-Volmer plots were fit into eqn4 to determine the K_{sv} values. Panels 4C and 4D are the category plots of the K_{sv} values for apoPgp and Pgp in the presence of AMPPNP, respectively. Table 2 shows the composition of 7-KC in proteoliposomes, and the corresponding K_{sv} values deduced from fitting the kinetic curves deduced by acrylamide quenching of tryptophan residues. Like CHL, increasing the composition of 7KC decreases the K_{sv} of Pgp. However, CHL appears to have a higher effect on the conformation of Pgp than 7KC.

4.5 DISCUSSION

The expression and function of Pgp involve multi-faceted mechanisms encompassing local and external conditions (Ahmed Juvalé et al., 2022; Robinson & Tiriveedhi, 2020). One of the local factors influencing the function of Pgp is the lipid bilayer, with cholesterol being a prominent member. Sterols are relatively small, inflexible, and mostly hydrophobic molecules that significantly impact membrane fluidity. Previous findings have revealed an inverse association between cholesterol synthesis and the survival rate of patients on chemotherapy (Weinstein et al., 2013). A similar relationship has been observed in the rate of tumor progression in cancer patients (Kuzu et al., 2016; Weinstein et al., 2013). When sterols accumulate in a lipid bilayer, they create a tighter packing of fatty acid tails, forming gel-phase lipid conditions with solid-like characteristics. Even though, the coupling effect of CHL on the activity of membrane proteins, such as Pgp, is well established (dos Santos et al., 2007; Wen et al., 2018), the mechanism underlying this intricate relationship remain elusive. Despite numerous studies in the last decade targeted at exploring this mechanism, there has been limited and sometimes, contradictory. It is unsurprising that the synthesis, transport, or metabolites of the cholesterol homeostasis pathways have been targeted recently as options for controlling cancer development (Kuzu et al., 2016). Based on the available literature, it is likely that sterols affect the activity of Pgp via two mechanisms: (1) directly through the lipid-Pgp interactions and (2) indirectly through changes in the physical properties of the membrane that either activate the protein or enhance Pgp-substrate interactions.

The first mechanism is supported by studies suggesting that lipids in the lipid bilayer can be flip-flopped from one membrane leaflet to another (H. Jing et al., 2020; Sanyal & Menon, 2009). The energy barrier associated with this trans-bilayer migration of highly hydrophobic molecules with

polar and charged heads makes this mechanism unlikely. Nonetheless, this seemingly energetically costly process is widespread in cells and is crucial in various essential cellular functions, including cell growth, signaling, and maintenance. (Anglin et al., 2009; Sanyal & Menon, 2009; Sharom, 2011a). Several factors, including the action of some transmembrane proteins, have been shown to facilitate rapid bidirectional flip-flop of lipids between the membrane leaflets (Devaux et al., 2008; Nakao et al., 2016). In fact, in the absence of transmembrane facilitators of trans-bilayer motion, flip-flop times have been reported to be very slow (Sanyal & Menon, 2009). Several ATP-independent and -dependent transmembrane proteins (Devaux et al., 2008; Rajasekharan & Gummadi, 2011), including Pgp (Garrigues et al., 2002; Thangapandian et al., 2020), are central to the flip-flop of lipids in the bilayer. Three possible binding sites of CHL on Pgp have been identified, all located within clefts on the surface of the protein (Domicevica et al., 2018). To date, no available data suggest the binding of CHL to the central cavity of the Pgp. The lack of presence in the central binding cavity raises the question of whether CHL justifies the role as a ‘genuine’ Pgp substrate since typical substrates primarily bind to one of the binding sites within the protein rather than on its surface. The affinity of Pgp for ATP, as determined from the ATPase kinetic curves, in the presence of 7KC was more than double that of 25HC and approximately three times as much as observed for CHL. On the other hand, the V_{max} values estimated from the ATPase kinetic curves for CHL, 7KC, and 25HC were similar. Since the sterols tested in this study differ primarily by their polar residues (Fig.1), one can infer that the polar residues play a significant role in interactions between Pgp and derivatives of cholesterol. This inference is supported by earlier *in silico* reports suggesting that the regions around the polar heads of CHL show significant interactions with Pgp (Domicevica et al., 2018; Thangapandian et al., 2020). Studies probing the flip-flop of CHL by Pgp revealed that all observable flipping events occurred within a region

formed by helices TM1, TM2, and TM11. During this process, the amino acid residues Tyr49, Tyr126, and Met928 were identified as crucial in the interaction between derivatives of cholesterol and Pgp (Thangapandian et al., 2020). Based on the characteristics of these amino acid residues, hydrogen bonding would be the most likely mode of interaction with the polar heads of cholesterol. Note that this does not negate the potential hydrophobic interactions that may occur between cholesterol derivatives and the amino acid residues within the cleft on the surface of the protein where cholesterol binds. The number and location of the polar residues in cholesterol derivatives may explain the level of stimulation observed in Pgp-mediated ATP activity. The disparity in the observed rate of stimulation of basal ATPase activity by sterols under study can be attributed to the additional polar moieties. Specifically, the presence of extra carbonyl residues on the polar head of 7KC enhances its interaction with Pgp, thereby promoting both the translocation of Pgp between the lipid leaflets and the rate of ATP hydrolysis. In contrast, the presence of polar moiety on the hydrophobic tail of CHL, as seen in 25HC, results in a minor but statistically insignificant increase in affinity of Pgp for ATP.

The mechanisms through which cholesterol may indirectly stimulate the activity of Pgp include the potential modulation of drug binding and the alteration of the membrane, resulting in the structural stability of the protein. Lipids play an essential role in the binding and translocation of the substrates by Pgp (Gayet et al., 2005; Loo & Clarke, 2016; Tran et al., 2023). One of the most accepted models for the catalytic activity of Pgp is the hydrophobic vacuum pump (Sharom, 2014), supported by various x-ray crystallography, electron microscopy, and various biochemical and biophysical assays (Hennessy & Spiers, 2007; J. Li et al., 2014; Sharom, 2014). The hydrophobic vacuum pump model propose that Pgp-mediated transport of substrates occur through a combination of passive diffusion and active transport mechanisms. Hydrophobic substrates enter

the lipid bilayer before being translocated to the central cavity of Pgp. Once inside this central cavity, substrates can engage with drug-binding pockets, triggering conformational changes in the protein. These conformational changes may facilitate the translocation of substrates across the membrane. Since Pgp substrates primarily engage with the central binding cavity of the protein in the inter-leaflet space of the lipid bilayer, substrate must be able to communicate with the membrane, partition into the bilayer, and subsequently interact with the central binding pocket within the transmembrane regions of the transporter. The presence of cholesterol in the lipid bilayer has been shown to lower the free energy associated with partitioning Pgp substrates into the membrane (Subramanian, Schumann-Gillett, et al., 2016). The enhanced transport activity observed with increasing cholesterol concentration in the lipid bilayer (Yan et al., 2020) may be attributed to the preferential accumulation of Pgp substrates in cholesterol-rich membrane regions. Unsurprisingly, most Pgp substrates have distinct, highly hydrophobic characteristics (Aller et al., 2009; C. Chen et al., 2018; Seelig, 2020). Moreover, the removal of cholesterol resulted in the alteration of the localization and loss of the function of Pgp (Kamau et al., 2005). One such relatively hydrophobic molecule that stimulates Pgp-mediated ATPase activity at lower substrate concentration (30 μ M) is verapamil (Ledwitch, Gibbs, et al., 2016). Of the three cholesterol derivatives under study, the presence of CHL in the lipid bilayer had the highest stimulatory effect on verapamil-induced ATPase activity, followed by 7KC. The introduction of the polar residues on the cholesterol likely reduced the predominantly hydrophobic interactions between the membrane and substrates. CHL, which is relatively more hydrophobic, interacts better with verapamil, enabling better partitioning into the lipid. This sequence of events allowed increased interaction between verapamil and residues at the central cavity of the protein, which resulted in higher Pgp-mediated ATP hydrolysis. Given the strong coupling between the rate of Pgp-mediated

ATP hydrolysis and transport (Li-Blatter et al., 2012), we can infer that the presence of CHL in liposomes stimulates the translocation of verapamil by Pgp at a relatively higher rate. The highest level of ATPase activity stimulation occurred when the composition of the sterols in liposomes was 20 percent. Whereas the compositions of CHL and 7KC in the lipid bilayer higher than 20 percent resulted in a slight reduction from the highest stimulatory levels, the effect of 25HC tapered at around 40 percent in liposomes. This phenomenon is probably due to polar residue on the tail of the 25HC, which may influence its interaction with surrounding lipids with highly hydrophobic tails.

A widely accepted model for the catalytic cycle of Pgp involves alternation between a predominantly open-inward orientation, where the protein is exposed to the cytosol with the nucleotide-binding domains (NBDs) separated, and an open-outward orientation, where the protein faces the extracellular environment with the NBDs in proximity. Note that several orientations lie between these two extreme orientations. The overall stability of the membrane proteins in the lipid bilayer is influenced by the ability to attain a stable minimum energy state (White & Wimley, 1999). Factors such as the interaction of the peptide chains with each other and the lipid bilayer hydrocarbon core, the bilayer interface, and water interactions play crucial roles in ensuring membrane proteins' stability and functionality (White & Wimley, 1999). By serving as a co-solvent for membrane proteins, cholesterol and its derivatives influence the stability and folding of proteins via the changes in the membrane (Y. Song et al., 2014). Nature is biased toward increasing entropy in a system. Therefore, an ensemble of Pgp would largely favor widely open-inward conformation. This phenomenon may create an orientation where the nucleotide-binding domains are relatively too wide apart, creating an unsurmountable barrier for transport. We hypothesize that cholesterol and its derivatives reduce the fluidity of the plasma

membrane, thereby maintaining the distance between the NBDs within a favorable reach. This phenomenon reduces the conformational barrier between the two NBDs and ensures the ability of the protein to transition through the conformations (open-inward to open-outward) required for transport. This phenomenon probably accounts for the observed inactive state of the protein in a membrane-free environment (Loo & Clarke, 2016). This theory aligns closely with the findings from our structural studies, which involved Pgp reconstituted into liposomes with varying compositions of the tested sterol (Fig. 4). Increasing percentage of either CHL (Fig. 4) or 7KC (Fig. 5) in the absence or presence of saturating AMPPNP resulted in concentration-dependent decrease in the K_{sv} values of Pgp fluorescence, suggesting a shift in the conformation of Pgp toward a reducing distance between the NBDs. The reduction of the conformational barrier due to the distance between the NBDs creates the conducive orientation needed for nucleotide binding and the subsequent shift of the protein to an open-outward conformation. A similar mechanism has been observed in the relationship between substrates that traditionally stimulate Pgp-mediated ATPase and the corresponding conformational changes induced in the protein (Pan & Aller, 2018; Zoghbi et al., 2017). At 0 percent composition in liposomes, AMPPNP showed a relatively lower effect on the conformation of Pgp. However, as the composition of sterols increased, the effect of the AMPPNP also increased. This relationship peaked at 20 percent for CHL, with an approximately 50 percent decrease in the K_{sv} value of Pgp. For 7KC, conformational shift induced by AMPPNP peaked at 30 percent composition in liposomes, likely due to the subtle influence of 7KC on the fluidity of the membrane compared to CHL. Previous *in silico* studies have shown that the presence of the carbonyl group on 7KC results in a more tilted orientation within the membrane compared to CHL, leading to significant adverse effects on lipid packing and orientation (Ayee &

Levitan, 2021; Kulig et al., 2015). These differential impacts on the packing of the lipid bilayer potentially account for the disparate Pgp conformational shifts observed.

In summary, sterols in the lipid bilayer have diverse effects on Pgp function, impacting cancer onset, progression, and chemotherapy outcomes. CHL and its derivatives interact with Pgp on its surface, flip-flopping between membrane leaflets, leading to increased basal Pgp-mediated ATPase activity. Additionally, sterols indirectly affect Pgp by altering membrane properties, including fluidity, which, in turn, influences protein conformation. 7KC exhibits higher basal Pgp activity, likely due to increased interaction with the protein and enhanced flip-flop activity. In contrast, CHL, being more hydrophobic than other sterols, promotes the partitioning of hydrophobic ligands into the interleaflet space, where the protein's central binding cavity is located. CHL's less tilted orientation further impacts membrane rigidity, favoring a transport-favorable Pgp conformation. Therefore, while 7KC may exhibit higher basal ATPase activity, CHL primarily influences lipid bilayer characteristics and modulates Pgp's interaction with its predominantly hydrophobic substrates.

4.6 FIGURES

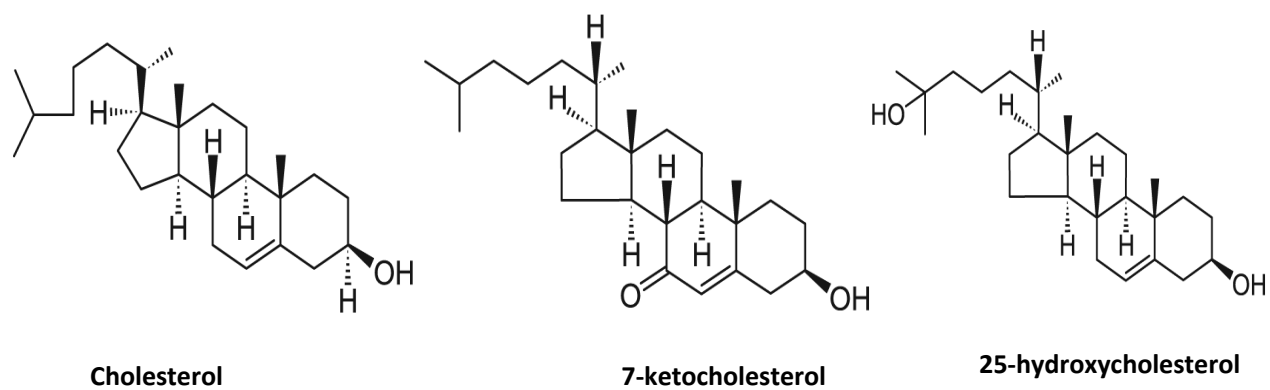


Figure 4.1 The structures cholesterol and some of the common oxidized derivatives

Figure 4.1. The structures cholesterol and some of the common oxidized derivatives

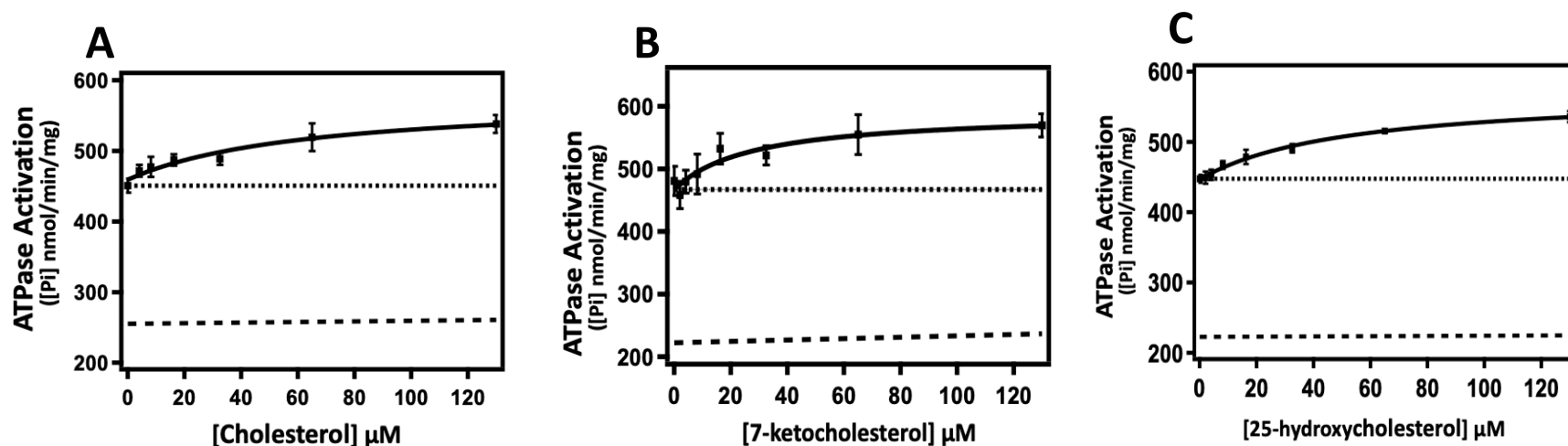


Figure 4.2 The effect of cholesterol and its derivatives on Pgp-mediated ATP hydrolysis

Figure 4.2. The effect of cholesterol and its derivatives on Pgp-mediated ATP hydrolysis. Ligand-induced Pgp-coupled ATPase activity in the presence of varying concentrations of (A) Cholesterol (closed squares), (B) 7-ketocholesterol (closed squares), and (C) 25-hydroxycholesterol (closed squares). Shown in each panel is the basal ATPase activity and the presence of 200 μM of vanadate. Each data point represents the average of 3 independent experiments, and the error bars shown represent the standard deviation.

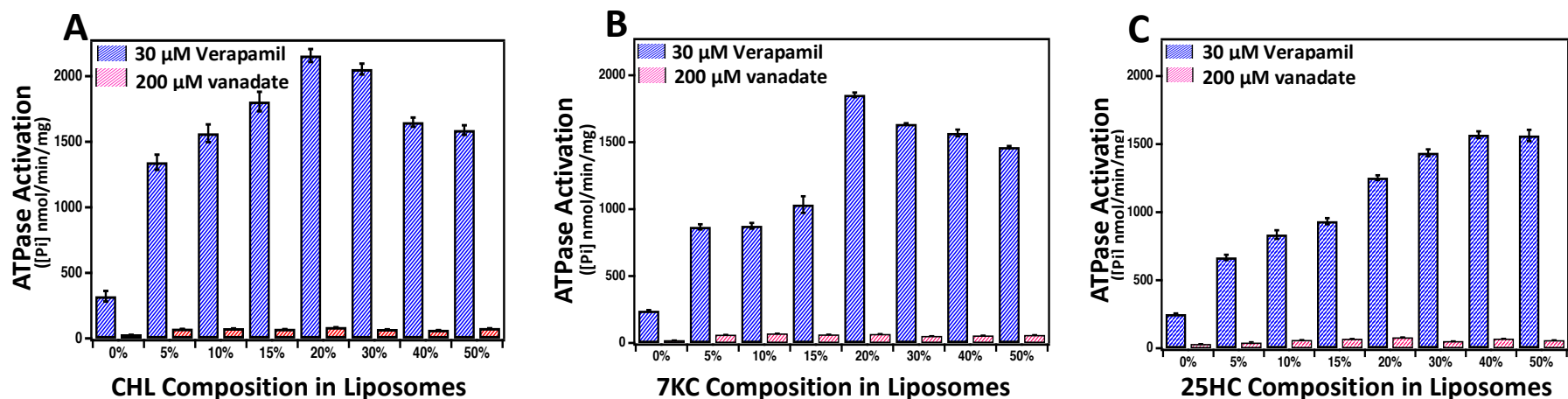


Figure 4.3 The effect of the derivatives of cholesterol on verapamil-stimulated Pgp-mediated ATP hydrolysis

Figure 4.3. The effect of the derivatives of cholesterol on verapamil-stimulated Pgp-mediated ATP hydrolysis. Verapamil-induced ATPase activity of Pgp reconstituted into liposomes consisting of varying compositions (A) CHL (blue stripes), (B) 7KC (blue stripes), and (C) 25HC (blue stripes). The average ATPase activity in the presence of 200 μ M of vanadate (red stripes) is shown for each condition. Each data point represents the average of at least 3 independent experiments, and the error bars shown represent the standard deviation.

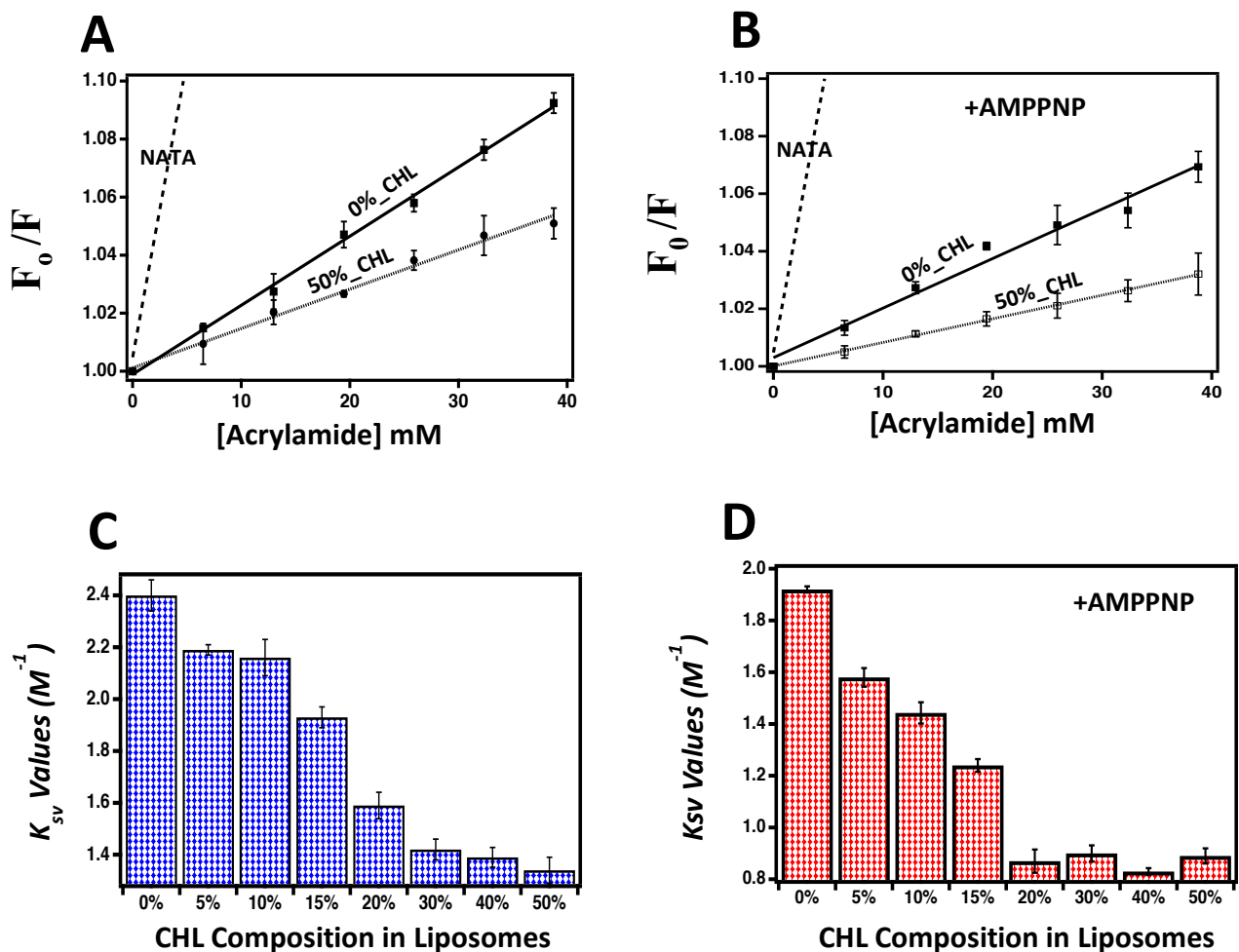


Figure 4.4 The effect of cholesterol (CHL) composition in liposomes on the conformation of Pgp probed by acrylamide quenching of protein fluorescence

Figure 4.4. The effect of cholesterol (CHL) composition in liposomes on the conformation of Pgp probed by acrylamide quenching of protein fluorescence. The Stern-Volmer plot of Pgp reconstituted into liposome with 0% (closed square) and 50% (open square) in the (A) absence and (B) presence of saturating (3.2 mM) AMPPNP. The K_{sv} values calculated from the Stern-Volmer plots are shown for the absence (C) and (D) presence of saturating AMPPNP. The data points and error bars represent the mean and standard deviation, respectively, for at least three independent experiments.

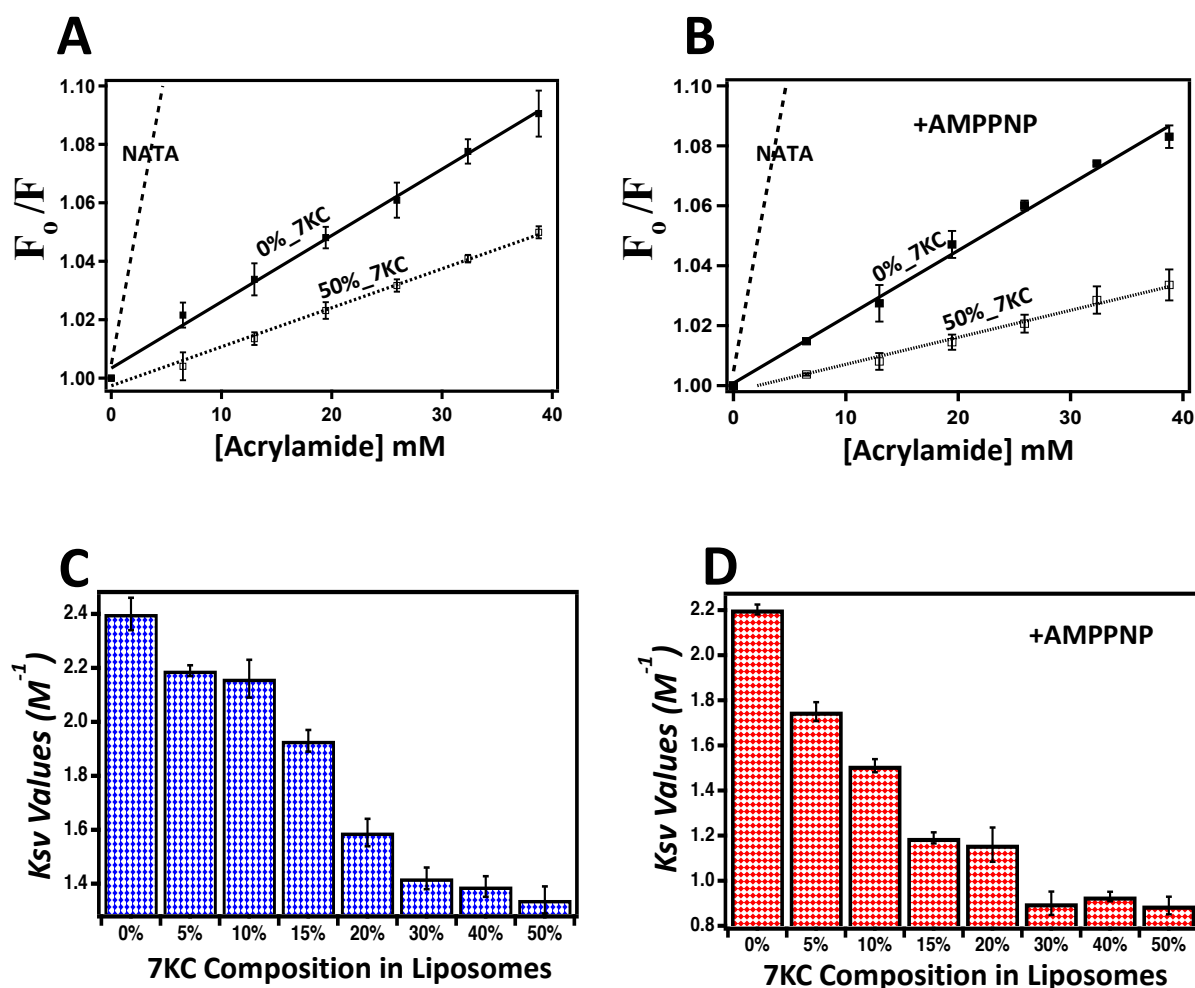


Figure 4.5 The impact of 7-ketocholesterol (7KC) composition within liposomes on Pgp conformation

Figure 4.5 The impact of 7-ketocholesterol (7KC) composition within liposomes on Pgp conformation, investigated through acrylamide quenching of protein fluorescence. Panels (A) and (B) represent the Stern-Volmer plots for Pgp reconstituted into liposomes containing 0% (closed square) and 50% (open square) cholesterol, both in the absence and presence of saturating (3.2 mM) AMPPNP, respectively. (C) and (D) Bar charts of the Ksv values derived from the Stern-Volmer plots for Pgp fluorescence in the presence and absence of saturating AMPPNP. The data points represent the mean values, with error bars indicating the standard deviation, based on results obtained from a minimum of three independent experiments.

4.7 TABLES

Table 4.1. The kinetic parameters and the corresponding averages deduced by fitting the effect of the CHL, 7KC, and 25HC on the rate of verapamil-stimulated Pgp-mediated ATPase activity Figure 3.

Composition of Cholesterol Derivatives in Liposomes	CHL (Pgp ATPase) nmolmin⁻¹mg⁻¹	7KC (Pgp ATPase) nmolmin⁻¹mg⁻¹	25HC (Pgp ATPase) nmolmin⁻¹mg⁻¹
0%	322 ± 41	274 ± 61	251 ± 12
5%	1344 ± 58	894 ± 35	667 ± 19
10%	1564 ± 68	854 ± 29	835 ± 31
15%	1807 ± 74	990 ± 63	934 ± 22
20%	2158 ± 49	1801 ± 63	1253 ± 17
30%	2055 ± 41	1652 ± 22	1436 ± 25
40%	1650 ± 32	1609 ± 48	1569 ± 24
50%	1589 ± 36	1508 ± 65	1564 ± 42

Table 4.2.

The K_{sv} value, obtained through fitting the Stern-Volmer plot of Pgp fluorescence, was determined for Pgp reconstituted into liposomes with varying cholesterol compositions (Fig. 4).

Cholesterol composition in Liposome	ApoPgp K_{sv} Values (μM^{-1})	+AMPpnp K_{sv} Values (μM^{-1})
0%	2.40 ± 0.06	1.92 ± 0.012
5%	2.19 ± 0.02	1.58 ± 0.036
10%	2.16 ± 0.07	$1,443 \pm 0.041$
15%	1.93 ± 0.04	1.24 ± 0.025
20%	1.59 ± 0.051	0.87 ± 0.045
30%	1.42 ± 0.04	0.9 ± 0.031
40%	1.39 ± 0.038	0.83 ± 0.013
50%	1.34 ± 0.05	0.89 ± 0.029

Table 3. The K_{sv} value extracted from the Stern-Volmer plot of Pgp reconstituted into liposomes with various 7KC compositions (Fig.5)

7-ketocholesterol composition in Liposome	ApoPgp K_{sv} Values (μM^{-1})	+AMPpnp K_{sv} Values (μM^{-1})
0%	2.494 ± 0.081	$2,203 \pm 0.022$
5%	2.24 ± 0.072	1.75 ± 0.042
10%	1.93 ± 0.047	1.51 ± 0.029
15%	1.9 ± 0.061	1.19 ± 0.025
20%	1.64 ± 0.074	1.16 ± 0.076
30%	1.47 ± 0.070	0.9 ± 0.052
40%	1.49 ± 0.057	0.93 ± 0.021
50%	1.447 ± 0.053	0.89 ± 0.039

CHAPTER 5

SYNOPSIS OF DISCUSSION AND FUTURE DIRECTIONS

5.1 SUMMARY

Pgp, discovered in the 1970s (Juliano & Ling, 1976), has a high evolutionary spectrum, spanning from bacteria to humans (Davidson & Chen, 2004; El-Awady et al., 2017). This widespread occurrence of the protein across diverse species underscores its importance for survival. In eukaryotes, Pgp is predominantly localized in the cell membrane, where it acts as a gatekeeper. It is unsurprising that this transporter is particularly abundant in tissues regularly exposed to potentially harmful substances, such as the liver, kidneys, intestines, and physiological barriers such as the blood-brain and blood-testis (Amin, 2013; Y. Li et al., 2010). Its strategic localization allows it to control the entry and exit of various molecules, including drugs and toxins. In addition to its protective role, Pgp has been implicated in several physiological processes, including bile secretion (Tian et al., 2007) and immune response (Ahmed Juvalé et al., 2022; Tanner et al., 2013). Studies revealed that Pgp could pump a wide range of drugs out of cells, making it a potent protector against toxic substances and conferring resistance to multiple drugs (Elmeliegy et al., 2020; Research, 2021). The expression and activity of Pgp can be influenced by a variety of intrinsic and extrinsic factors, and understanding these factors is crucial for comprehending drug resistance, pharmacokinetics, and the potential for therapeutic interventions. The expression levels of Pgp are influenced by factors such as age (Warrington et al., 2004), hormones (Brayboy et al., 2018), enzymes (Benderra et al., 2004; Linn et al., 1996), inflammatory mediators (Saib & Delavenne, 2021), dietary and environmental compounds (Nabekura et al., 2005), and transcription factors (Braconi et al., 2022; Elmeliegy et al., 2020; Pilotto Heming et al., 2022),

epigenetics (Henrique et al., 2013); alterations in any of which could result in the generation of a disease phenotype.

5.1.1 The Modulating Effect of Cholesterol Derivatives on The Activity Of P-Glycoprotein

The activity of Pgp can also be influenced by various extrinsic factors, with the membrane environment being one of the most significant (Ahmed Juvala et al., 2022; Robinson & Tiriveedhi, 2020). Pgp is embedded within the lipid bilayer of cell membrane and has been shown to be nearly inactive without the lipid environment. The unique characteristics of the lipid bilayer significantly influence the stability and function of Pgp via the alteration of membrane fluidity, lipid rafts, and membrane potential. Variations in lipid composition, such as the presence of cholesterol or phospholipids, can alter the characteristics of the lipid bilayer, thereby influencing Pgp activity (dos Santos et al., 2007; Wen et al., 2018). In this dissertation, the unique role of cholesterol in modulating the Pgp activity was investigated. Three primary mechanisms were proposed for the lipid bilayer's unique role in modulating the activity of Pgp.

The first mechanism involves the flip-flop of the lipids between membrane leaflets, which is energetically costly but crucial for cellular functions (Anglin et al., 2009; Sanyal & Menon, 2009; Sharom, 2011a). Transmembrane proteins, including Pgp, facilitate this process (Garrigues et al., 2002; Thangapandian et al., 2020). All the potential binding sites of CHL derivatives proposed have been within clefts on the surface of Pgp (Domicевичa et al., 2018), suggesting that CHL does not likely interact with the central binding cavity of the protein. The affinity of Pgp for ATP induced by the presence of sterols depend on the type of sterol, with polar residues playing a significant role. 7-ketocholesterol (7KC), a cholesterol derivative with an extra polar residue on the head, induced higher Pgp-ATP affinity than the parent compound (Fig. 4.2). Another derivative of CHL, 25-hydroxycholesterol (25HC), induced slightly higher but

statistically insignificant Pgp-ATP affinity. Studies indicate that the interactions between Pgp and cholesterol derivatives involve hydrogen bonding and occur within the region formed by specific helices, with amino acid residues Tyr49, Tyr126, and Met928 proposed to be highly involved in the interaction (Thangapandian et al., 2020). Thus, it is likely that the extra polar residue on the head of 7KC enables better interactions with the protein and enhances Pgp-mediated flip-flopping. This development likely accounts for the relatively enhanced Pgp-mediated ATP activity observed for 7KC in these studies (Fig. 4.2). 25HC, on the other hand, has a polar residue on the tail of CHL, resulting in energetically unfavorable flip-flopping (Fig. 4.2C).

Cholesterol and its oxidized derivatives can also indirectly modulate the activity of Pgp by mediating substrates partitioning into the inter-leaflet space of the lipid bilayer, where the ligand binding cavity of the Pgp is located (Subramanian, Schumann-Gillett, et al., 2016). Lipids, including cholesterol, play a crucial role in how Pgp interacts with substrates. Lipids in the membrane, due to their relative hydrophobic characteristics, enhance the activity of Pgp by reducing the energy required for substrates to enter the membrane, making it easier for substrates to accumulate in the membrane, especially cholesterol-rich regions. This observation is evidenced by cholesterol's relatively high stimulatory effect on verapamil-induced ATPase basal activity compared to oxidized cholesterol derivatives (Fig. 4). Cholesterol, with a lesser number of polar residues, increases the hydrophobic interactions with substrates, allowing for better partitioning of substrates into the lipid bilayer. An increased Pgp-substrate interaction within the central cavity of the protein leads to increased ATP hydrolysis and substrate translocation to the extracellular environment.

Maintaining the stability and functionality of membrane proteins like Pgp relies on attainment of a stable minimum energy state within the lipid bilayer (White & Wimley, 1999). An ensemble of Pgp molecules typically favors a widely open-inward conformation due to the tendency to increase entropy. However, this orientation creates a conformational barrier for transport, evidenced by the inactivity of the isolated Pgp in a membrane-free environment (Sharom, 2014). Factors such as peptide chain interactions, interactions with the lipid bilayer, the bilayer interface, and water interactions are crucial for this stability. Cholesterol derivatives, acting as co-solvents, influence the protein's stability and folding by altering the membrane environment. Cholesterol and its derivatives achieve this feat by reducing membrane fluidity, which helps maintain an optimal NBDs distance that allows Pgp to transition between the necessary conformations for effective transport to occur. Increasing CHL or 7KC percentages in liposomes led to concentration-dependent shifts in Pgp conformation towards a reduced NBD distance (Fig. 4). This reduction in the conformational barrier allows for nucleotide binding and the subsequent shift to an open-outward conformation, facilitating transport.

5.1.2 Challenges in Addressing Pgp-Induced Multidrug Resistance

Due to the multifaceted roles Pgp plays in both health and disease contexts, addressing Pgp-induced multidrug resistance (MDR) poses considerable challenges. Alterations in Pgp expression and function can have detrimental effects on pivotal cellular functions such as immune response, cholesterol metabolism, cell differentiation, migration, and intrinsic defense mechanisms safeguarding the body against xenobiotics and harmful substances (Ahmed Juvalé et al., 2022). Substantial evidence demonstrates a correlation between Pgp expression levels and drug effectiveness (Robinson & Tiriveedhi, 2020). This correlation suggests a logical basis for developing Pgp inhibitors and CRISPR/Cas-mediated knockdowns as potential treatments for

cancer. Even though the inhibition of Pgp function may be able to reduce drug resistance, it could also potentially introduce additional health complications. In fact, there are currently no approved Pgp inhibitors for cancer treatment (Nanayakkara et al., 2018). Recently, attempts have been made to repurpose already FDA-approved drugs that have been shown to inhibit the activity of Pgp. However, both pre-clinical and clinical studies that explored these potential remedies have not been successful (Karthika et al., 2022). For instance, verapamil, a well-studied Pgp inhibitor that effectively inhibits Pgp in pre-clinical studies, failed in clinical studies due to limited effectiveness, drug-drug interactions, and intolerable toxicity (Karthika et al., 2022; Lai et al., 2020). Similar developments have been observed in the other FDA-approved drugs re-purposed as Pgp inhibitors. In addition, a proposed Pgp knockdown appears promising in mitigating the action of Pgp but can also make patients more vulnerable to environmental toxicant. The impact of combinational therapy that utilizes Pgp inhibitors in chemotherapy on fetal development in pregnant women cannot be overstated.

One viable strategy involves designing therapeutic drugs that can circumvent the activity of Pgp by being either non-substrate or poor substrate. However, developing a new therapeutic agent could be laborious and very expensive (Wouters et al., 2020, pp. 2009–2018), not to add the extra constraint of ensuring the drug candidates are at least very poor Pgp substrate. A better approach would be redesigning current drugs as a strategy for cancer treatment. This approach offers the benefit of a well-known toxicity profile and mechanism of action, minimizing unexpected setbacks in clinical development. From this cautiously optimistic perspective, we investigated the mechanisms behind the Pgp-mediated transport of certain widely recognized chemotherapeutic agents, whose efficacy has been limited by the action of Pgp. We also proposed potential moieties on the alkaloids to which structure-activity-relationship (SAR) data-driven

modifications could pave the way for the next generation of effective and cost-efficient chemotherapeutic agents. One of the most common classes of chemotherapeutic agents are the alkaloids, which include camptothecin analogs: irinotecan (IRT), SN-38, and topotecan (TPT); vinca alkaloids: vinblastine (VBL) and vinorelbine (VRL) and vincristine (VCR); Taxanes: paclitaxel and docetaxel, and podo phyllotoxins: etoposide and teniposide (*Types of Chemotherapy Drugs* | SEER Training, n.d.)

5.1.3 Drug-Induced Conformational Dynamics of P-Glycoprotein Underlies the Transport of Camptothecin Analogs

Camptothecin analogs (CPTs), a member of the plant-based chemotherapeutic alkaloids, consist of essential anticancer drugs such as topotecan (TPT), irinotecan (IRT), and SN-38 (F. Li et al., 2017; Tesaro et al., 2019). These drugs primarily target topoisomerase I, an essential enzyme involved in DNA replication. CTPs binds to and stabilizes topoisomerase-I-DNA complexes at the replication fork and induce potentially deadly breaks in the DNA strands (Laco, 2011; F. Li et al., 2017). These drugs are commonly used as first- and second-line treatments for various types of cancer, including colorectal carcinoma (F. Li et al., 2017). IRT is a prodrug activated by carboxylesterase to SN-38, which is estimated to be as much as 1000 times more potent than the prodrug (Parvez et al., 2021). Despite sharing a common structural base, the CPTs show significant differences in their pharmaceutical and pharmacological properties. Pre-clinical studies correlate the rate of intracellular accumulation of the CTPs with Pgp-mediated transports, suggesting that Pgp plays a crucial role in reducing the levels of these drugs below therapeutically relevant concentrations required to be effective as anticancer agents (Hirose et al., 2012; Houghton et al., 1995; Lin et al., 2011; Salphati et al., 2009; Y. Zhao et al., 2019). The associated systemic toxicity of the CPTs in pre- and clinical studies is primarily due to the higher than necessary physiologically

relevant dose required to achieve therapeutic effect. These unfortunate developments are due to the action of Pgp and, to a lesser extent, other transporter proteins. Given the vital role of CPTs in chemotherapy, it's not surprising that researchers are developing and testing other analogs, like 9-amino camptothecin, in pre-clinical and clinical studies. Unfortunately, these analogs might face the same challenges as existing antitumor agents if they are significantly transported by Pgp.

Even though the Pgp-mediated efflux rates of the CPTs are well established (Lin et al., 2011; Salphati et al., 2009), the mechanisms mediating this process remain elusive. In fact, even the functional groups necessary for Pgp-CPT interactions are yet to be identified. In this study, a conformationally-gated model was used to explain how Pgp transports the CPTs (Fig. 2.7). Pgp, in the absence or presence of substrates, consists of an ensemble with diverse conformational states. To simplify our model, Pgp was categorized into three conformations: 'open-inward', 'intermediate', and 'open-outward'. In the open-inward conformation, nucleotide-binding domains (NBDs) are relatively apart, with the drug-binding pockets exposed to the inside of the cell. Conversely, open-outward conformation is when NBDs are in proximity, and the drug-binding sites are accessible from outside the cell. The 'intermediate' conformation falls in between open-inward and open-outward conformations.

Conformational studies using acrylamide quenching of intrinsic protein fluorescence revealed that TPT caused a significant decrease in the K_{sv} value of apoPgp, akin to the effect induced by AMPPNP (Fig. 2.4). Since Pgp in the presence of AMPPNP (Gibbs et al., 2018; Sonveaux et al., 1999) has been shown to be in a predominantly open-outward conformation, we can infer that TPT shifts Pgp into an open-inward conformation. AFM imaging showed a significant increase in the population of Pgp within the tallest heights when TPT was added, which supports our hypothesis that TPT shifts Pgp into a predominantly open-outward conformation. SN-

38 and IRT caused a minimal decrease in the K_{sv} value, implying a lesser influence on the conformation of Pgp. AFM height distribution histograms showed that SN-38 slightly affected the tallest and shortest Pgp populations. At the same time, IRT caused a relatively more significant shift toward the population of Pgp with widely open-inward conformations (Fig. 2.6).

Our observations suggest that drug-induced protein conformational changes influence the Pgp-mediated transport of CPTs. TPT causes the most significant conformational change in Pgp, reducing the NBDs separating distance and the conformational barrier. The binding of SN-38 to Pgp leads to an intermediate state, between open-inward and open-outward, with a slight reduction in the conformational barrier, resulting in limited SN-38 transport. On the other hand, IRT shifts Pgp closer to the open-inward conformation and is poorly transported. Our structurally-gated model aligns with the available efflux ratio and biochemical data presented in this study. We propose that Pgp-mediated ATPase activation is influenced by ligand-induced conformational changes in Pgp, with the highest and lowest activation likely occurring with open-outward and open-inward Pgp conformations, respectively. The distance between the NBDs is a barrier affecting substrate transport, with substrates like TPT that mitigate this barrier translocated across the lipid bilayer faster. While the analysis of Pgp-ligand binding affinity indicates that TPT has the highest affinity for the protein, followed by SN-38, differences observed were minimal and are unlikely to be the primary driving mechanism for the differential transport rates reported for the CPTs. Interestingly, all the tested CPTs induce a similar open-outward conformation in Pgp when saturating AMPPNP is present, emphasizing the critical role of nucleotides in the protein's catalytic cycle.

The STDD NMR analyses reveal that, on average, TPT had a higher binding interaction with Pgp, which aligns with our binding affinity studies (Fig. 2.5). In addition, our study identified

specific functional moieties on TPT and IRT likely involved in their binding to Pgp. Modifying these functional groups based on the available structure-activity relationship could pave the way for developing improved drugs that are less susceptible to Pgp-mediated efflux. To our knowledge, this study is the first to identify the specific functional groups on TPT and IRT that likely interact with Pgp.

5.1.4 Exploring the Mechanisms Underlying Pgp-Mediated Transport of Vinca Alkaloids

The vinca alkaloids (VAs), initially derived from the *Catharanthus roseus* plant, are a class of alkaloids widely used as an anticancer agent (van Der Heijden et al., 2004). The VAs has also been used previously to manage conditions like diabetes and hypertension due to their hypoglycemic effects (Tyagi et al., 2010). VAs, used as antitumor medications, operate by binding to and interfering with the polymerization of microtubules. This disruption results in cell cycle arrest and cell death. VAs include the FDA-approved drugs like vincristine (VCR), vinblastine (VBL), vinorelbine (VRL), vindesine (VDS), and vinflunine (VFL) (Dhyani et al., 2022; Moudi et al., 2013). Despite their structural similarities (Fig. 3.1) and similar microtubule-drug affinity, the VAs show significant differences in their potency as an anticancer agent, a phenomenon that cannot that partition coefficient values cannot solely account for. There is a strong correlation between cellular retention and potency (Lobert et al., 1996; Ngan et al., 2000), with VCR showing the highest retention and efficacy, followed by VRL. On the contrary, continuous *in vitro* treatment with either VBL or VCR yielded comparable efficacy (Gout et al., 1984), implying that transmembrane transporters which reduces cellular concentrations may be implicated. *In vitro* studies indicate that Pgp significantly efflux VAs, with VBL exhibiting the highest rate followed by VRL (Lin et al., 2011). These Pgp-mediated efflux rates were significantly reduced in the presence of a Pgp inhibitor, Zosuquidar (Lin et al., 2011). However, the exact mechanisms behind

these differences in Pgp-mediated transport rates remain unclear, emphasizing the need for a comprehensive understanding of the transport mechanism associated. The results from these studies could lay the foundation for developing the next generation of VAs from existing chemotherapeutic agents.

The collective data from *in silico* (Iqbal et al., 2022) and biophysical studies (Aanismaa & Seelig, 2007; Romsicki & Sharom, 1999) suggest two distinct binding sites for VAs on Pgp: a high-affinity site within the central cavity and a low-affinity site near the protein-lipid interface. VAs primarily bind to the high-affinity central cavity sites at low substrate concentrations, leading to enhanced ATP binding and hydrolysis. Of the three compounds tested, VBL exhibits the highest Pgp-mediated transport rate at lower concentrations (Fig. 3.2), aligning with experimental transport rate data (Ferguson & Cass, 1985; Lin et al., 2011; Pierré et al., 1992). Pgp-mediated stimulation of ATPase activity and transport is influenced by ligand binding affinity and ligand-induced conformational changes in the protein. VBL shows a higher binding affinity for Pgp compared to VRL and VCR (Fig. 3.3). The differences in binding affinity are linked to specific moieties in VAs, particularly the C₃₀ moiety (see Fig 3.1), which distinguishes VBL from VCR. Surprisingly, at the tested concentration (10 μ M), VAs do not significantly impact Pgp's conformation (Fig.3.4), suggesting that the differential binding affinity observed for the VAs is the rate-limiting factor for Pgp-mediated transport rather than conformational changes. Note that a subtle conformational dynamics in peptide segments observed in a previous study (Chai et al., 2022), may play a role in facilitating transport. For instance, alterations in transmembrane helix 12 (TM12), crucial for the communication between the drug-binding cavity and NBDs, has been observed upon VA binding to Pgp (Chai et al., 2022)..

At higher drug concentrations, VAs appear to bind to a low-affinity site near the interface between the protein and the lipid bilayer, leading to inhibitory effects on ATPase activity (Fig. 3.2). Two theories can explain this inhibition. The first theory suggests that VAs interact with the same binding sites as endogenous substrates like cholesterol but at a slower rate, which would appear to inhibit ATPase activity because Pgp-mediated ATPase activity encompasses both basal and ligand-induced activation. The second theory suggests that VAs behave similarly to certain inhibitors, acting as substrates at low concentrations and inhibitors at higher concentrations. This theory is more substantiated by existing literature. When VAs bind to the second site, they likely impede the required protein reorientation required for substrate transport, as observed in some Pgp inhibitors. However, VAs have a relatively lower affinity for the second binding site, making them less potent as Pgp inhibitors. The inhibitory site is surrounded by multiple transmembrane helices and features phenylalanine residues that interact with bound ligands, suggesting that weak hydrophobic interactions may be the predominant mode of interaction. The binding of Pgp inhibitors to this site restricts specific transmembrane helices, which prevent substrate translocation and obstruct the access tunnel and vestibule of the protein, preventing the necessary shift when a substrate binds to the central cavity. The K_i values for VAs suggest that VCR has the highest inhibitory potential at higher substrate concentrations, followed by VBL and VRL, and this order of affinity at the inhibitory binding site is the reverse of the stimulatory binding site.

At 150 μM , VAs induced slight but statistically insignificant differences in the conformation of Pgp (Fig. 3.4). This observation aligns with the results of AFM imaging, which assessed the distribution of Pgp heights within the cytosolic region when exposed to 150 μM VAs (Fig. 3.6). VBL and VRL had a minimal impact on Pgp height distribution, while VCR shifted the distribution slightly to the left, increasing the population of the protein in widely open-inward

conformations. Pgp-mediated transport is often dependent on ligand-induced conformational changes, and transport is typically minimal when the protein is in a predominantly widely open-inward conformation. Based on these findings, it can be inferred that VCR has the highest inhibitory influence on Pgp-mediated transport at saturating substrate concentrations compared to the other two drugs. These subtle conformational changes, detected with highly sensitive techniques like AFM, maybe the underlying structural mechanism mediating the transport of VAs by Pgp.

At low concentrations (10 μ M) and saturating AMPPNP, VAs induced a conformational shift in Pgp similar to the effect of AMPPNP alone. This similarity may be due to the low substrate concentration compared to the saturating concentration of AMPPNP used. However, at saturating drug (150 μ M) and AMPPNP (3.2 mM) concentrations, VAs induced a conformational shift in Pgp that was intermediate between open-inward and open-outward (Fig. 3.4). Notably, VCR induced a lesser conformational shift in the presence of AMPPNP compared to VBL and VRL, while there was no significant difference between the effects of VBL and VRL.

There is a need for the next generation of VAs with improved response rates and reduced toxicity. Vinflunine, a third-generation VA with lower toxicity, shows promise but is less potent, requiring significantly higher concentrations for similar effects as VCR. Developing new VA derivatives has been challenging, but identifying functional groups involved in Pgp-VAs interactions offers hope for rational drug design. STDD NMR analysis revealed that specific functional groups on VAs are involved in Pgp interactions, with protons linked to the methyl groups playing significant roles (See Fig. 1 and Fig. 3.5). Modifying hydrogen bond acceptor (HBA) and hydrophobic groups in regions implicated in Pgp-drug recognition, as identified by STDD NMR, could lead to the development of next-generation VAs capable of evading the

transporter. This promising approach may come with functional limitations. Combining our understanding of functional groups with structure-activity relationship (SAR) studies can guide the search for more effective next-generation VA derivatives.

To conclude, at low substrate concentrations, VAs bind to the stimulatory site within Pgp's central cavity, causing subtle changes in TM 12 that promote ATP binding and an open-outward conformation facilitate transport. However, VAs occupy both stimulatory and inhibitory sites at high substrate concentrations, resulting in an intermediate conformation that inhibits ATP hydrolysis and transport. Our study offers insights into the structure of Pgp-mediated VA efflux and suggests potential functional groups involved in substrate recognition. This information, combined with SAR studies, could inform the design of next-generation VAs with improved potency, reduced systemic toxicity, and cost effective.

5.2 FUTURE DIRECTIONS

5.2.1 Leveraging *In Silico* and Biophysical Studies to Explore the Structural Dynamics of Pgp

The understanding of the conformational dynamics of Pgp is a fundamental aspect of unraveling the mechanisms underlying Pgp-mediated transport, particularly concerning the alkaloids explored in this dissertation. While this study has provided valuable insights into the structural behavior of Pgp, future studies capable of measuring subtle changes in the distance between the NBDs holds the promise of further elucidating the intricacies of Pgp dynamics. The determination of NBDs' separating distances is critical for comprehending the conformational changes that occur during the transport cycle of Pgp. One such technique involves computational studies. *In silico* studies have proven to be a valuable tool for exploring the dynamics of biomolecules over time. Building upon the biophysical studies presented in this study, extensive and long duration simulations aimed at quantifying the distances between the NBDs with time could be employed. Longer simulation times can capture subtle changes in NBD distances that may have been missed in the biophysical investigations presented in this dissertation. Moreover, advanced computational methodologies, such as enhanced sampling techniques (e.g., metadynamics, accelerated molecular dynamics), can be employed to accelerate the exploration of conformational space and uncover transient states relevant to Pgp dynamics. Furthermore, to investigate the influence of membrane composition on Pgp behavior, the simulation of Pgp must be done in a protein embedded in lipid bilayers with varying compositions of cholesterol. This will not only foster accurate mimicking of the protein in a natural environment, but it could also provide insight into how the membrane environment impacts NBD distances, substrate recognition, and transport kinetics.

Promising biophysical methods, including advanced nuclear magnetic resonance (NMR) experiments, electron paramagnetic resonance (EPR) spectroscopy, and Förster resonance energy transfer (FRET), chemical cross-linking assays, if optimized, could help shed more light on the structural dynamism of Pgp and offer insight into the role the membrane plays in modulating the activity of Pgp. The journey to a comprehensive understanding the structural dynamics of Pgp is still an ongoing venture, and future research that infuse experimental and computational could shed light on the intricate mechanism underlying the activity of this vital transporter protein, ultimately contributing to the development of more effective therapeutic strategies and overcoming drug resistance.

5.2.2 Rational Drug Design for Pgp-Evading Derivatives of the Alkaloids

Building upon the foundation provided by this study regarding the functional groups of camptothecin analogs and vinca alkaloids that likely interact with Pgp, studies aimed at developing novel derivatives capable of evading this transporter have the potential to revolutionize this field. The approach of modifying existing drugs to enhance their efficacy and overcome multidrug resistance presents a cost-effective and expedited pathway that can significantly benefit from the wealth of already available data on these drugs. By utilizing available structural-activity relationship studies, studies can make informed decisions about which functional groups to target for modification and avoid the potential loss of functionality that may occur. Rational structural modifications can involve adding, substituting, or removing specific chemical moieties to fine-tune the drugs' interaction with Pgp. In addition, computational tools can help predict the impact of structural changes on the binding affinity and transport kinetics of these derivatives with Pgp, a phenomenon that can play a pivotal role in guiding a rational drug design. This approach allows for the rapid evaluation of numerous potential modifications, streamlining the drug design process.

Once a number of possible candidates has been established, a high-throughput screening assays could be used in testing the candidates to identify lead compounds with lower Pgp-substrate affinity that retains therapeutic efficacy. The synergy between computational predictions and experimental screening can expedite the discovery of promising drug candidates. Successful candidates emerging from preclinical studies should undergo clinical evaluation that assess the safety and efficacy of the modified derivatives in human patients. These trials may include individuals with drug-resistant cancers, where a drug's potential to overcome multidrug resistance can be thoroughly examined. Thus, future research in rational drug design for Pgp-evading derivatives of camptothecin analogs and vinca alkaloids holds great promise for addressing drug resistance in cancer and beyond. By strategically modifying existing drugs, leveraging computational tools, conducting high-throughput screening, and advancing candidates through preclinical and clinical evaluations, we can potentially revolutionize treatment options for patients facing multidrug resistance.

6.0 REFERENCE

1. Aanismaa, P., & Seelig, A. (2007). P-Glycoprotein kinetics measured in plasma membrane vesicles and living cells. *Biochemistry*, 46(11), 3394–3404. <https://doi.org/10.1021/bi0619526>
2. Abbott, N. J. (2014). Anatomy and physiology of the blood–brain barriers. In *Drug Delivery to the Brain* (pp. 3–21). Springer.
3. *ABCB1 ATP binding cassette subfamily B member 1 [Homo sapiens (human)]—Gene—NCBI*. (n.d.). Retrieved April 9, 2023, from <https://www.ncbi.nlm.nih.gov/gene/5243>
4. Abu-Qare, A. W., Elmasry, E., & Abou-Donia, M. B. (2003). A role for P-glycoprotein in environmental toxicology. *J Toxicol Environ Health B Crit Rev*, 6(3), 279–288.
5. Agarwal, S., Uchida, Y., Mittapalli, R. K., Sane, R., Terasaki, T., & Elmquist, W. F. (2012). Quantitative proteomics of transporter expression in brain capillary endothelial cells isolated from P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp), and P-gp/Bcrp knockout mice. *Drug Metabolism and Disposition*, 40, 1164–1169.
6. Ahmed Juvala, I. I., Abdul Hamid, A. A., Abd Halim, K. B., & Che Has, A. T. (2022). P-glycoprotein: New insights into structure, physiological function, regulation and alterations in disease. *Heliyon*, 8(6), e09777. <https://doi.org/10.1016/j.heliyon.2022.e09777>
7. Akbar, S. MD., Sreeramulu, K., & Sharma, H. C. (2016). Tryptophan fluorescence quenching as a binding assay to monitor protein conformation changes in the membrane of intact mitochondria. *Journal of Bioenergetics and Biomembranes*, 48(3), 241–247. <https://doi.org/10.1007/s10863-016-9653-0>

8. Alam, A., Kowal, J., Broude, E., Roninson, I., & Locher, K. P. (2019). Structural insight into substrate and inhibitor discrimination by human P-glycoprotein. *Science*, 363(6428), 753–756. <https://doi.org/10.1126/science.aav7102>
9. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). The Lipid Bilayer. In *Molecular Biology of the Cell. 4th edition*. Garland Science. <https://www.ncbi.nlm.nih.gov/books/NBK26871/>
10. Aller, S. G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P. M., Trinh, Y. T., Zhang, Q., Urbatsch, I. L., & Chang, G. (2009). Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science (New York, N.Y.)*, 323(5922), 1718–1722. <https://doi.org/10.1126/science.1168750>
11. Almazroo, O. A., Miah, M. K., & Venkataramanan, R. (2017). Drug metabolism in the liver. *Clinics in Liver Disease*, 21(1), 1–20. <https://doi.org/10.1016/j.cld.2016.08.001>
12. Al-Shawi, M. K., & Omote, H. (2005). The remarkable transport mechanism of P-glycoprotein; a multidrug transporter. *Journal of Bioenergetics and Biomembranes*, 37(6), 489–496. <https://doi.org/10.1007/s10863-005-9497-5>
13. Amawi, H., Sim, H.-M., Tiwari, A. K., Ambudkar, S. V., & Shukla, S. (2019). ABC Transporter-Mediated Multidrug-Resistant Cancer. *Advances in Experimental Medicine and Biology*, 1141, 549–580. https://doi.org/10.1007/978-981-13-7647-4_12
14. Ambudkar, S. V., Cardarelli, C. O., Pashinsky, I., & Stein, W. D. (1997). Relation Between the Turnover Number for Vinblastine Transport and for Vinblastine-stimulated ATP Hydrolysis by Human P-glycoprotein. *Journal of Biological Chemistry*, 272(34), 21160–21166. <https://doi.org/10.1074/jbc.272.34.21160>

15. Ambudkar, S. V., Kimchi-Sarfaty, C., Sauna, Z. E., & Gottesman, M. M. (2003). P-glycoprotein: From genomics to mechanism. *Oncogene*, 22(47), 7468–7485. <https://doi.org/10.1038/sj.onc.1206948>
16. Amin, Md. L. (2013). P-glycoprotein Inhibition for Optimal Drug Delivery. *Drug Target Insights*, 7, 27–34. <https://doi.org/10.4137/DTI.S12519>
17. Andersen, V., Østergaard, M., Christensen, J., Overvad, K., Tjønneland, A., & Vogel, U. (2009). Polymorphisms in the xenobiotic transporter Multidrug Resistance 1 (MDR1) and interaction with meat intake in relation to risk of colorectal cancer in a Danish prospective case-cohort study. *BMC Cancer*, 9(1), 407. <https://doi.org/10.1186/1471-2407-9-407>
18. Ando, K., Shah, A. K., Sachdev, V., Kleinstiver, B. P., Taylor-Parker, J., Welch, M. M., Hu, Y., Salgia, R., White, F. M., Parvin, J. D., Ozonoff, A., Rameh, L. E., Joung, J. K., & Bharti, A. K. (2017). Camptothecin resistance is determined by the regulation of topoisomerase I degradation mediated by ubiquitin proteasome pathway. *Oncotarget*, 8(27), 43733–43751. <https://doi.org/10.18632/oncotarget.16376>
19. Anglicheau, D., Pallet, N., Rabant, M., Marquet, P., Cassinat, B., Méria, P., Beaune, P., Legendre, C., & Thervet, E. (2006). Role of P-glycoprotein in cyclosporine cytotoxicity in the cyclosporine–sirolimus interaction. *Kidney International*, 70(6), 1019–1025. <https://doi.org/10.1038/sj.ki.5001649>
20. Anglin, T. C., Brown, K. L., & Conboy, J. C. (2009). Phospholipid Flip-flop Modulated by Transmembrane Peptides WALP and Melittin. *Journal of Structural Biology*, 168(1), 37–52. <https://doi.org/10.1016/j.jsb.2009.06.001>

21. Annaert, P. P., Turncliff, R. Z., Booth, C. L., Thakker, D. R., & Brouwer, K. L. R. (2001). P-Glycoprotein-Mediated In Vitro Biliary Excretion in Sandwich-Cultured Rat Hepatocytes. *Drug Metabolism and Disposition*, 29(10), 1277–1283.
22. Aryal, M., Fischer, K., Gentile, C., Gitto, S., Zhang, Y.-Z., & McDannold, N. (2017). Effects on P-Glycoprotein Expression after Blood-Brain Barrier Disruption Using Focused Ultrasound and Microbubbles. *PLOS ONE*, 12(1), e0166061. <https://doi.org/10.1371/journal.pone.0166061>
23. Asaumi, R., Nunoya, K., Yamaura, Y., Taskar, K. S., & Sugiyama, Y. (2022). Robust physiologically based pharmacokinetic model of rifampicin for predicting drug–drug interactions via P-glycoprotein induction and inhibition in the intestine, liver, and kidney. *CPT: Pharmacometrics & Systems Pharmacology*, 11(7), 919–933. <https://doi.org/10.1002/psp4.12807>
24. Association, T. S. of C. R. of O. M. of C. A., Center, B. C. E. C. of N. C. Q. C., & Institute, B. C. I. (2022). Expert consensus on the clinical application of PI3K/AKT/mTOR inhibitors in the treatment of advanced breast cancer. *Cancer Innovation*, 1(1), 25–54. <https://doi.org/10.1002/cai2.10>
25. Atkinson, D. E., Greenwood, S. L., Sibley, C. P., Glazier, J. D., & Fairbairn, L. J. (2003). Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer. *American Journal of Physiology-Cell Physiology*, 285(3), C584–C591. <https://doi.org/10.1152/ajpcell.00418.2002>
26. Ayee, M. A. A., & Levitan, I. (2021). Lipoprotein-Induced Increases in Cholesterol and 7-Ketocholesterol Result in Opposite Molecular-Scale Biophysical Effects on Membrane Structure. *Frontiers in Cardiovascular Medicine*, 8, 715932. <https://doi.org/10.3389/fcvm.2021.715932>
27. Bachner, M., & De Santis, M. (2008). Vinflunine in the treatment of bladder cancer. *Therapeutics and Clinical Risk Management*, 4(6), 1243–1253.

28. Bai, J., Swartz, D. J., Protasevich, I. I., Brouillette, C. G., Harrell, P. M., Hildebrandt, E., Gasser, B., Mattanovich, D., Ward, A., Chang, G., & Urbatsch, I. L. (2011). A gene optimization strategy that enhances production of fully functional P-glycoprotein in *Pichia pastoris*. *PLOS ONE*, 6(8), e22577. <https://doi.org/10.1371/journal.pone.0022577>
29. Barbieri, A., Thonghin, N., Shafi, T., Prince, S. M., Collins, R. F., & Ford, R. C. (2021). Structure of ABCB1/P-Glycoprotein in the Presence of the CFTR Potentiator Ivacaftor. *Membranes*, 11(12), Article 12. <https://doi.org/10.3390/membranes11120923>
30. Bársony, O., Szalóki, G., Türk, D., Tarapcsák, S., Gutay-Tóth, Z., Bacsó, Z., Holb, I. J., Székvölgyi, L., Szabó, G., Csanády, L., Szakács, G., & Goda, K. (2016). A single active catalytic site is sufficient to promote transport in P-glycoprotein. *Scientific Reports*, 6. <https://doi.org/10.1038/srep24810>
31. Barthomeuf, C., Grassi, J., Demeule, M., Fournier, C., Boivin, D., & Béliveau, R. (2005). Inhibition of P-glycoprotein transport function and reversion of MDR1 multidrug resistance by enniadin. *Cancer Chemotherapy and Pharmacology*, 56(2), 173–181. <https://doi.org/10.1007/s00280-004-0914-y>
32. Basit, A., Radi, Z., Vaidya, V. S., Karasu, M., & Prasad, B. (2019). Kidney Cortical Transporter Expression across Species Using Quantitative Proteomics. *Drug Metabolism and Disposition*, 47(8), 802–808. <https://doi.org/10.1124/dmd.119.086579>
33. Bauer, M., Tournier, N., & Langer, O. (2019). Imaging P-Glycoprotein Function at the Blood–Brain Barrier as a Determinant of the Variability in Response to Central Nervous System Drugs. *Clinical Pharmacology & Therapeutics*, 105(5), 1061–1064. <https://doi.org/10.1002/cpt.1402>
34. Baylin, S. B., & Jones, P. A. (2016). Epigenetic Determinants of Cancer. *Cold Spring Harbor Perspectives in Biology*, 8(9), a019505. <https://doi.org/10.1101/cshperspect.a019505>

35. Ben Saad, A., Bruneau, A., Mareux, E., Lapalus, M., Delaunay, J.-L., Gonzales, E., Jacquemin, E., Aït-Slimane, T., & Falguières, T. (2021). Molecular Regulation of Canalicular ABC Transporters. *International Journal of Molecular Sciences*, 22(4), 2113. <https://doi.org/10.3390/ijms22042113>
36. Benadiba, M., & Maor, Y. (2016). Importance of ABC Transporters in Drug Development. *Current Pharmaceutical Design*, 22(38), 5817–5829. <https://doi.org/10.2174/1381612822666160810120359>
37. Benderra, Z., Faussat, A.-M., Sayada, L., Perrot, J.-Y., Chaoui, D., Marie, J.-P., & Legrand, O. (2004). Breast Cancer Resistance Protein and P-Glycoprotein in 149 Adult Acute Myeloid Leukemias. *Clinical Cancer Research*, 10(23), 7896–7902. <https://doi.org/10.1158/1078-0432.CCR-04-0795>
38. Benet, L. Z., & Cummins, C. L. (2001). The drug efflux–metabolism alliance: Biochemical aspects. *Advanced Drug Delivery Reviews*, 50, S3–S11.
39. Bhuiyan, M., Petropoulos, S., Gibb, W., & Matthews, S. G. (2012). Sertraline Alters Multidrug Resistance Phosphoglycoprotein Activity in the Mouse Placenta and Fetal Blood–Brain Barrier. *Reproductive Sciences*, 19(4), 407–415. <https://doi.org/10.1177/1933719111424438>
40. Binkhathlan, Z., & Lavasanifar, A. (2013). P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: Current status and future perspectives. *Current Cancer Drug Targets*, 13(3), 326–346. <https://doi.org/10.2174/15680096113139990076>
41. Blakeslee, J. J., Bandyopadhyay, A., Lee, O. R., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S. N., Cheng, Y., Bouchard, R., Adamec, J., Geisler, M., Nagashima, A., Sakai, T., Martinoia, E., Friml, J., Peer, W. A., & Murphy, A. S. (2007). Interactions among PIN-FORMED

and P-Glycoprotein Auxin Transporters in Arabidopsis. *The Plant Cell*, 19(1), 131–147.
<https://doi.org/10.1105/tpc.106.040782>

42. Blundell, C., Tess, E. R., Schanzer, A. S. R., Coutifaris, C., Su, E. J., Parry, S., & Huh, D. (2016). A microphysiological model of the human placental barrier. *Lab on a Chip*, 16(16), 3065–3073. <https://doi.org/10.1039/c6lc00259e>

43. Bonito, C. A., Ferreira, R. J., Ferreira, M.-José. U., Durães, F., Sousa, E., Gillet, J.-P., Cordeiro, M. N. D. S., & dos Santos, D. J. V. A. (2023). Probing the Allosteric Modulation of P-Glycoprotein: A Medicinal Chemistry Approach Toward the Identification of Noncompetitive P-Gp Inhibitors. *ACS Omega*, 8(12), 11281–11287. <https://doi.org/10.1021/acsomega.2c08273>

44. Borgnia, M. J., Eytan, G. D., & Assaraf, Y. G. (1996). Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem*, 271(6), 3163–3171.

45. Bossennec, M., Di Roio, A., Caux, C., & Ménétrier-Caux, C. (2018). MDR1 in immunity: Friend or foe? *OncotImmunology*, 7(12), e1499388. <https://doi.org/10.1080/2162402X.2018.1499388>

46. Boumendjel, A., Boutonnat, J., & Robert, J. (2009). *ABC Transporters and Multidrug Resistance* (15684779). John Wiley & Sons.

47. Bournissen, F. G., Moretti, M. E., Juurlink, D. N., Koren, G., Walker, M., & Finkelstein, Y. (2009). Polymorphism of the MDR1/ABCB1 C3435T drug-transporter and resistance to anticonvulsant drugs: A meta-analysis. *Epilepsia*, 50(4), 898–903. <https://doi.org/10.1111/j.1528-1167.2008.01858.x>

48. Braconi, L., Teodori, E., Riganti, C., Coronello, M., Nocentini, A., Bartolucci, G., Pallecchi, M., Contino, M., Manetti, D., Romanelli, M. N., Supuran, C. T., & Dei, S. (2022). New Dual P-

Glycoprotein (P-gp) and Human Carbonic Anhydrase XII (hCA XII) Inhibitors as Multidrug Resistance (MDR) Reversers in Cancer Cells. *Journal of Medicinal Chemistry*, 65(21), 14655–14672. <https://doi.org/10.1021/acs.jmedchem.2c01175>

49. Brayboy, L. M., Knapik, L. O., Long, S., Westrick, M., & Wessel, G. M. (2018). Ovarian hormones modulate multidrug resistance transporters in the ovary. *Contraception and Reproductive Medicine*, 3(1), 26. <https://doi.org/10.1186/s40834-018-0076-7>

50. Broccatelli, F., Carosati, E., Neri, A., Frosini, M., Goracci, L., Oprea, T. I., & Cruciani, G. (2011). A Novel Approach for Predicting P-glycoprotein (ABCB1) Inhibition Using Molecular Interaction Fields. *Journal of Medicinal Chemistry*, 54(6), 1740–1751. <https://doi.org/10.1021/jm101421d>

51. Bukowski, K., Kciuk, M., & Kontek, R. (2020). Mechanisms of Multidrug Resistance in Cancer Chemotherapy. *International Journal of Molecular Sciences*, 21(9), 3233. <https://doi.org/10.3390/ijms21093233>

52. Callaghan, R., Luk, F., & Bebawy, M. (2014). Inhibition of the multidrug resistance P-glycoprotein: Time for a change of strategy? *Drug Metabolism and Disposition*, 42(4), 623–631. <https://doi.org/10.1124/dmd.113.056176>

53. Callen, D. F., Baker, E., Simmers, R. N., Seshadri, R., & Roninson, I. B. (1987). Localization of the human multiple drug resistance gene, MDR1, to 7q21.1. *Human Genetics*, 77(2), 142–144. <https://doi.org/10.1007/BF00272381>

54. Campbell, K., Zappas, A., Bunz, U., Thio, Y. S., & Bucknall, D. G. (2012). Fluorescence quenching of a poly(para-phenylene ethynylenes) by C60 fullerenes. *Journal of Photochemistry and Photobiology A: Chemistry*, 249, 41–46. <https://doi.org/10.1016/j.jphotochem.2012.08.015>

55. Cao, Y., Shi, Y., Cai, Y., Hong, Z., & Chai, Y. (2020). The Effects of Traditional Chinese Medicine on P-Glycoprotein–Mediated Multidrug Resistance and Approaches for Studying the Herb–P-Glycoprotein Interactions. *Drug Metabolism and Disposition*, 48(10), 972–979. <https://doi.org/10.1124/dmd.120.000050>
56. Casares, D., Escribá, P. V., & Rosselló, C. A. (2019). Membrane Lipid Composition: Effect on Membrane and Organelle Structure, Function and Compartmentalization and Therapeutic Avenues. *International Journal of Molecular Sciences*, 20(9). <https://doi.org/10.3390/ijms20092167>
57. Cavaco, M. C., Pereira, C., Kreutzer, B., Gouveia, L. F., Silva-Lima, B., Brito, A. M., & Videira, M. (2017). Evading P-glycoprotein mediated-efflux chemoresistance using Solid Lipid Nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, 110, 76–84. <https://doi.org/10.1016/j.ejpb.2016.10.024>
58. Chai, A. B., Callaghan, R., & Gelissen, I. C. (2022). Regulation of P-Glycoprotein in the Brain. *International Journal of Molecular Sciences*, 23(23), Article 23. <https://doi.org/10.3390/ijms232314667>
59. Chaillou, S., Durant, J., Garraffo, R., Georgenthum, E., Roptin, C., Clevenbergh, P., Dunais, B., Mondain, V., Roger, P. M., & Dellamonica, P. (2002). Intracellular concentration of protease inhibitors in HIV-1-infected patients: Correlation with MDR-1 gene expression and low dose of ritonavir. *HIV Clinical Trials*, 3(6), 493–501. <https://doi.org/10.1310/0873-bvdp-akay-445u>
60. Chan, G. N. Y., Patel, R., Cummins, C. L., & Bendayan, R. (2013). Induction of P-Glycoprotein by Antiretroviral Drugs in Human Brain Microvessel Endothelial Cells. *Antimicrobial Agents and Chemotherapy*, 57(9), 4481–4488. [https://doi.org/10.1128/AAC.00486-](https://doi.org/10.1128/AAC.00486-13)

61. Chan, L. M., Lowes, S., & Hirst, B. H. (2004). The ABCs of drug transport in intestine and liver: Efflux proteins limiting drug absorption and bioavailability. *European Journal of Pharmaceutical Sciences*, 21, 25–51.
62. Chawla, A., Repa, J. J., Evans, R. M., & Mangelsdorf, D. J. (2001). Nuclear Receptors and Lipid Physiology: Opening the X-Files. *Science*, 294(5548), 1866–1870. <https://doi.org/10.1126/science.294.5548.1866>
63. Chen, C., Lee, M.-H., Weng, C.-F., & Leong, M. K. (2018). Theoretical Prediction of the Complex P-Glycoprotein Substrate Efflux Based on the Novel Hierarchical Support Vector Regression Scheme. *Molecules*, 23(7), Article 7. <https://doi.org/10.3390/molecules23071820>
64. Chen, K. G., Lacayo, N. J., Durán, G. E., Wang, Y., Bangs, C. D., Rea, S., Kovacs, M., Cherry, A. M., Brown, J. M., & Sikic, B. I. (2002). Preferential expression of a mutant allele of the amplified MDR1 (ABCB1) gene in drug-resistant variants of a human sarcoma. *Genes, Chromosomes and Cancer*, 34(4), 372–383. <https://doi.org/10.1002/gcc.10067>
65. Chen, M. L., Sun, A., Cao, W., Eliason, A., Mendez, K. M., Getzler, A. J., Tsuda, S., Diao, H., Mukori, C., Bruno, N. E., Kim, S. Y., Pipkin, M. E., Koralov, S. B., & Sundrud, M. S. (2020). Physiological expression and function of the MDR1 transporter in cytotoxic T lymphocytes. *Journal of Experimental Medicine*, 217(5), e20191388. <https://doi.org/10.1084/jem.20191388>
66. Chen, Q., Bian, Y., & Zeng, S. (2014). Involvement of AP-1 and NF- κ B in the Up-regulation of P-gp in Vinblastine Resistant Caco-2 Cells. *Drug Metabolism and Pharmacokinetics*, 29(2), 223–226. <https://doi.org/10.2133/dmpk.DMPK-13-SH-068>
67. Chen, Y., & Liu, L. (2012). Modern methods for delivery of drugs across the blood–brain barrier. *Advanced Drug Delivery Reviews*, 64, 640–665.

68. Chen, Z., Han, F., Du, Y., Shi, H., & Zhou, W. (2023). Hypoxic microenvironment in cancer: Molecular mechanisms and therapeutic interventions. *Signal Transduction and Targeted Therapy*, 8(1), Article 1. <https://doi.org/10.1038/s41392-023-01332-8>
69. Chifflet, S., Torriglia, A., Chiesa, R., & Tolosa, S. (1988). A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: Application to lens ATPases. *Analytical Biochemistry*, 168(1), 1–4. [https://doi.org/10.1016/0003-2697\(88\)90002-4](https://doi.org/10.1016/0003-2697(88)90002-4)
70. Choo, E. F., Leake, B., Wandel, C., Imamura, H., Wood, A. J., Wilkinson, G. R., & Kim, R. B. (2000). Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, 28(6), 655–660.
71. Chu, C.-Y., Henderson, D. J., & Parmeter, C. F. (2017). On discrete Epanechnikov kernel functions. *Computational Statistics & Data Analysis*, 116, 79–105. <https://doi.org/10.1016/j.csda.2017.07.003>
72. Chufan, E. E., Kapoor, K., Sim, H.-M., Singh, S., Talele, T. T., Durell, S. R., & Ambudkar, S. V. (2013). Multiple Transport-Active Binding Sites Are Available for a Single Substrate on Human P-Glycoprotein (ABCB1). *PLOS ONE*, 8(12), e82463. <https://doi.org/10.1371/journal.pone.0082463>
73. Chufan, E. E., Sim, H.-M., & Ambudkar, S. V. (2015). Molecular basis of the polyspecificity of P-glycoprotein (ABCB1): Recent biochemical and structural studies. *Advances in Cancer Research*, 125, 71–96. <https://doi.org/10.1016/bs.acr.2014.10.003>

74. Chung, F. S., Santiago, J. S., Jesus, M. F. M. D., Trinidad, C. V., & See, M. F. E. (2016). Disrupting P-glycoprotein function in clinical settings: What can we learn from the fundamental aspects of this transporter? *American Journal of Cancer Research*, 6(8), 1583–1598.
75. Cioni, P., & Strambini, G. B. (1998). Acrylamide Quenching of Protein Phosphorescence as a Monitor of Structural Fluctuations in the Globular Fold. *Journal of the American Chemical Society*, 120(45), 11749–11757. <https://doi.org/10.1021/ja9820543>
76. Cirrito, J. R., Deane, R., Fagan, A. M., Spinner, M. L., Parsadanian, M., Finn, M. B., Jiang, H., Prior, J. L., Sagare, A., & Bales, K. R. (2005). P-glycoprotein deficiency at the blood-brain barrier increases amyloid- β deposition in an Alzheimer disease mouse model. *Journal of Clinical Investigation*, 115, 3285.
77. Cisternino, S., Rousselle, C., Debray, M., & Scherrmann, J. M. (2004). In situ transport of vinblastine and selected P-glycoprotein substrates: Implications for drug-drug interactions at the mouse blood-brain barrier. *Pharm Res*, 21(8), 1382–1389.
78. Claasen, B., Axmann, M., Meinecke, R., & Meyer, B. (2005). Direct observation of ligand binding to membrane proteins in living cells by a saturation transfer double difference (STDD) NMR spectroscopy method shows a significantly higher affinity of integrin α IIb β 3 in native platelets than in liposomes. *Journal of the American Chemical Society*, 127, 916–919.
79. Clay, A. T., & Sharom, F. J. (2013). Lipid bilayer properties control membrane partitioning, binding, and transport of P-Glycoprotein substrates. *Biochemistry*, 52(2), 343–354. <https://doi.org/10.1021/bi301532c>
80. Clouser, A. F., & Atkins, W. M. (2022). Long Range Communication between the Drug-Binding Sites and Nucleotide Binding Domains of the Efflux Transporter ABCB1. *Biochemistry*, 61(8), 730–740. <https://doi.org/10.1021/acs.biochem.2c00056>

81. Corradi, V., Mendez-Villuendas, E., Ingólfsson, H. I., Gu, R.-X., Siuda, I., Melo, M. N., Moussatova, A., DeGagné, L. J., Sejdiu, B. I., Singh, G., Wassenaar, T. A., Delgado Magnero, K., Marrink, S. J., & Tieleman, D. P. (2018). Lipid–Protein Interactions Are Unique Fingerprints for Membrane Proteins. *ACS Central Science*, 4(6), 709–717. <https://doi.org/10.1021/acscentsci.8b00143>
82. Corradi, V., Sejdiu, B. I., Mesa-Galloso, H., Abdizadeh, H., Noskov, S. Yu., Marrink, S. J., & Tieleman, D. P. (2019). Emerging Diversity in Lipid–Protein Interactions. *Chemical Reviews*, 119(9), 5775–5848. <https://doi.org/10.1021/acs.chemrev.8b00451>
83. Crowley, E., O’Mara, M. L., Kerr, I. D., & Callaghan, R. (2010). Transmembrane helix 12 plays a pivotal role in coupling energy provision and drug binding in ABCB1. *The FEBS Journal*, 277(19), 3974–3985. <https://doi.org/10.1111/j.1742-4658.2010.07789.x>
84. Crowley, E., O’Mara, M. L., Reynolds, C., Tieleman, D. P., Storm, J., Kerr, I. D., & Callaghan, R. (2009). Transmembrane Helix 12 Modulates Progression of the ATP Catalytic Cycle in ABCB1. *Biochemistry*, 48(26), 6249–6258. <https://doi.org/10.1021/bi900373x>
85. D’Amelio, N., Aroulmoji, V., Toraldo, A., Sundaraganesan, N., & Anbarasan, P. M. (2012). Aggregation properties and structural studies of anticancer drug Irinotecan in DMSO solution based on NMR measurements. *Journal of Molecular Structure*, 1013, 26–35. <https://doi.org/10.1016/j.molstruc.2012.01.012>
86. Davidson, A. L., & Chen, J. (2004). ATP-Binding Cassette Transporters in Bacteria. *Annual Review of Biochemistry*, 73(1), 241–268. <https://doi.org/10.1146/annurev.biochem.73.011303.073626>

87. Davidson, A. L., Dassa, E., Orelle, C., & Chen, J. (2008). Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems. *Microbiology and Molecular Biology Reviews* : *MMBR*, 72(2), 317–364. <https://doi.org/10.1128/MMBR.00031-07>
88. Davies, T. G., Theodoulou, F. L., Hallahan, D. L., & Forde, B. G. (1997). Cloning and characterisation of a novel P-glycoprotein homologue from barley. *Gene*, 199(1–2), 195–202. [https://doi.org/10.1016/s0378-1119\(97\)00367-3](https://doi.org/10.1016/s0378-1119(97)00367-3)
89. Dawson, R. J. P., Hollenstein, K., & Locher, K. P. (2007). Uptake or extrusion: Crystal structures of full ABC transporters suggest a common mechanism. *Molecular Microbiology*, 65(2), 250–257. <https://doi.org/10.1111/j.1365-2958.2007.05792.x>
90. de Foresta, B., Vincent, M., Gallay, J., & Garrigos, M. (2010). Interaction with membrane mimics of transmembrane fragments 16 and 17 from the human multidrug resistance ABC transporter 1 (hMRP1/ABCC1) and two of their tryptophan variants. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1798(3), 401–414. <https://doi.org/10.1016/j.bbamem.2009.11.019>
91. De Lange, E. C. M., vd Berg, D. J., Bellanti, F., Voskuyl, R. A., & Syvänen, S. (2018). P-glycoprotein protein expression versus functionality at the blood-brain barrier using immunohistochemistry, microdialysis and mathematical modeling. *European Journal of Pharmaceutical Sciences*, 124, 61–70. <https://doi.org/10.1016/j.ejps.2018.08.022>
92. Dehghani-Ghahnaviyeh, S., Kapoor, K., & Tajkhorshid, E. (2021). Conformational changes of the nucleotide binding domains of P-glycoprotein induced by ATP hydrolysis. *FEBS Letters*, 595(6), 735–749. <https://doi.org/10.1002/1873-3468.13992>
93. Devaux, P. F., Herrmann, A., Ohlwein, N., & Kozlov, M. M. (2008). How lipid flippases can modulate membrane structure. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1778(7), 1591–1600. <https://doi.org/10.1016/j.bbamem.2008.03.007>

94. Dhyani, P., Quispe, C., Sharma, E., Bahukhandi, A., Sati, P., Attri, D. C., Szopa, A., Sharifi-Rad, J., Docea, A. O., Mardare, I., Calina, D., & Cho, W. C. (2022). Anticancer potential of alkaloids: A key emphasis to colchicine, vinblastine, vincristine, vindesine, vinorelbine and vincamine. *Cancer Cell International*, 22(1), 206. <https://doi.org/10.1186/s12935-022-02624-9>
95. di Nunzio, M. R., Wang, Y., & Douhal, A. (2013). Spectroscopy and dynamics of topotecan anti-cancer drug comprised within cyclodextrins. *Journal of Photochemistry and Photobiology A: Chemistry*, 266, 12–21. <https://doi.org/10.1016/j.jphotochem.2013.05.011>
96. Domicевичa, L., Koldsø, H., & Biggin, P. C. (2018). Multiscale molecular dynamics simulations of lipid interactions with P-glycoprotein in a complex membrane. *Journal of Molecular Graphics and Modelling*, 80, 147–156. <https://doi.org/10.1016/j.jmglm.2017.12.022>
97. Dong, C., Wu, J., Chen, Y., Nie, J., & Chen, C. (2021). Activation of PI3K/AKT/mTOR Pathway Causes Drug Resistance in Breast Cancer. *Frontiers in Pharmacology*, 12, 628690. <https://doi.org/10.3389/fphar.2021.628690>
98. dos Santos, S. M., Weber, C.-C., Franke, C., Müller, W. E., & Eckert, G. P. (2007). Cholesterol: Coupling between membrane microenvironment and ABC transporter activity. *Biochemical and Biophysical Research Communications*, 354(1), 216–221. <https://doi.org/10.1016/j.bbrc.2006.12.202>
99. Drescher, S., Schaeffeler, E., Hitzl, M., Hofmann, U., Schwab, M., Brinkmann, U., Eichelbaum, M., & Fromm, M. F. (2002). MDR1 gene polymorphisms and disposition of the P-glycoprotein substrate fexofenadine. *British Journal of Clinical Pharmacology*, 53(5), 526–534.
100. Drozdziak, M., Busch, D., Lapczuk, J., Müller, J., Ostrowski, M., Kurzawski, M., & Oswald, S. (2018). Protein Abundance of Clinically Relevant Drug-Metabolizing Enzymes in the

Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens. *Clinical Pharmacology & Therapeutics*, 104(3), 515–524. <https://doi.org/10.1002/cpt.967>

101. Dubrovay, Z., Háda, V., Béni, Z., & Szántay, C. (2013). NMR and mass spectrometric characterization of vinblastine, vincristine and some new related impurities – Part I. *Journal of Pharmaceutical and Biomedical Analysis*, 84, 293–308. <https://doi.org/10.1016/j.jpba.2012.08.019>

102. Duffin, J. (2000). Poisoning the spindle: Serendipity and discovery of the anti-tumor properties of the Vinca alkaloids. *Canadian Bulletin of Medical History = Bulletin Canadien D'histoire De La Medecine*, 17(1–2), 155–192. <https://doi.org/10.3138/cbmh.17.1.155>

103. Dufrêne, Y. F., Ando, T., Garcia, R., Alsteens, D., Martinez-Martin, D., Engel, A., Gerber, C., & Müller, D. J. (2017). Imaging modes of atomic force microscopy for application in molecular and cell biology. *Nature Nanotechnology*, 12(4), Article 4. <https://doi.org/10.1038/nnano.2017.45>

104. Dunn, B., & Wobbe, C. R. (2001). Preparation of protein extracts from yeast. *Curr Protoc Mol Biol, Chapter 13*, Unit13 13. <https://doi.org/10.1002/0471142727.mb1313s23>

105. Durmus, S., Hendriks, J. J. M. A., & Schinkel, A. H. (2015). Chapter one—Apical ABC Transporters and Cancer Chemotherapeutic Drug Disposition. In J. D. Schuetz & T. Ishikawa (Eds.), *Advances in Cancer Research* (Vol. 125, pp. 1–41). Academic Press. <https://doi.org/10.1016/bs.acr.2014.10.001>

106. Eckford, P. D., & Sharom, F. J. (2008). Functional characterization of Escherichia coli MsbA: interaction with nucleotides and substrates. *Journal of Biological Chemistry*, 283, 12840–12850. <https://doi.org/10.1074/jbc.M708274200>

107. Eckford, P. D., & Sharom, F. J. (2009). ABC efflux pump-based resistance to chemotherapy drugs. *Chemical Reviews*, 109, 2989–3011. <https://doi.org/10.1021/cr9000226>

108. Edwards, L., & Wanless, I. R. (2013). Mechanisms of liver involvement in systemic disease. *Best Practice & Research Clinical Gastroenterology*, 27(4), 471–483. <https://doi.org/10.1016/j.bpg.2013.08.002>
109. Egashira, M., Kawamata, N., Sugimoto, K., Kaneko, T., & Oshimi, K. (1999). P-Glycoprotein Expression on Normal and Abnormally Expanded Natural Killer Cells and Inhibition of P-Glycoprotein Function by Cyclosporin A and Its Analogue, PSC833. *Blood*, 93(2), 599–606. <https://doi.org/10.1182/blood.V93.2.599>
110. Eisenbraun, M. D., Mosley, R. L., Teitelbaum, D. H., & Miller, R. A. (2000). Altered development of intestinal intraepithelial lymphocytes in P-glycoprotein-deficient mice. *Developmental & Comparative Immunology*, 24(8), 783–795. [https://doi.org/10.1016/S0145-305X\(00\)00029-X](https://doi.org/10.1016/S0145-305X(00)00029-X)
111. El Hayek, M., Trad, O., & Jamil, A. (2005). Vincristine-induced urinary bladder paralysis. *Journal of Pediatric Hematology/Oncology*, 27(5), 286–287. <https://doi.org/10.1097/01.mph.0000165130.21539.a3>
112. El-Awady, R., Saleh, E., Hashim, A., Soliman, N., Dallah, A., Elrasheed, A., & Elakraa, G. (2017). The Role of Eukaryotic and Prokaryotic ABC Transporter Family in Failure of Chemotherapy. *Frontiers in Pharmacology*, 7. <https://www.frontiersin.org/article/10.3389/fphar.2016.00535>
113. Elkins, M. R., Sergeyev, I. V., & Hong, M. (2018). Determining Cholesterol Binding to Membrane Proteins by Cholesterol ¹³ C Labeling in Yeast and Dynamic Nuclear Polarization NMR. *Journal of the American Chemical Society*, 140(45), 15437–15449. <https://doi.org/10.1021/jacs.8b09658>

114. Elmeliegy, M., Vourvahis, M., Guo, C., & Wang, D. D. (2020). Effect of P-glycoprotein (P-gp) Inducers on Exposure of P-gp Substrates: Review of Clinical Drug–Drug Interaction Studies. *Clinical Pharmacokinetics*, 59(6), 699–714. <https://doi.org/10.1007/s40262-020-00867-1>
115. Emami Nejad, A., Najafgholian, S., Rostami, A., Sistani, A., Shojaeifar, S., Esparvarinha, M., Nedaeinia, R., Haghjooy Javanmard, S., Taherian, M., Ahmadlou, M., Salehi, R., Sadeghi, B., & Manian, M. (2021). The role of hypoxia in the tumor microenvironment and development of cancer stem cell: A novel approach to developing treatment. *Cancer Cell International*, 21(1), 62. <https://doi.org/10.1186/s12935-020-01719-5>
116. Ernest, S., & Bello-Reuss, E. (1998). P-glycoprotein functions and substrates: Possible roles of MDR1 gene in the kidney. *Kidney International. Supplement*, 65, S11-17.
117. Esser, L., Zhou, F., Pluchino, K. M., Shiloach, J., Ma, J., Tang, W., Gutierrez, C., Zhang, A., Shukla, S., Madigan, J. P., Zhou, T., Kwong, P. D., Ambudkar, S. V., Gottesman, M. M., & Xia, D. (2017). Structures of the Multidrug Transporter P-glycoprotein Reveal Asymmetric ATP Binding and the Mechanism of Polyspecificity♦. *The Journal of Biological Chemistry*, 292(2), Article 2. <https://doi.org/10.1074/jbc.M116.755884>
118. Evans, D. C., O'Connor, D., Lake, B. G., Evers, R., Allen, C., & Hargreaves, R. (2003). Eletriptan metabolism by human hepatic CYP450 enzymes and transport by human P-glycoprotein. *Drug Metabolism and Disposition*, 31, 861–869.
119. Evans, R. M., & Mangelsdorf, D. J. (2014). Nuclear Receptors, RXR, and the Big Bang. *Cell*, 157(1), 255–266. <https://doi.org/10.1016/j.cell.2014.03.012>
120. Eyal, S., Chung, F. S., Muzi, M., Link, J. M., Mankoff, D. A., Kaddoumi, A., O'Sullivan, F., Hebert, M. F., & Unadkat, J. D. (2009). Simultaneous PET imaging of P-glycoprotein inhibition in multiple tissues in the pregnant non-human primate. *Journal of Nuclear Medicine : Official*

Publication, Society of Nuclear Medicine, 50(5), 798–806.

<https://doi.org/10.2967/jnumed.108.059360>

121. Fantini, J., & Barrantes, F. (2013). How cholesterol interacts with membrane proteins: An exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains. *Frontiers in Physiology*, 4. <https://www.frontiersin.org/articles/10.3389/fphys.2013.00031>

122. Feng, X., & Xie, H.-G. (2016). *Applying Pharmacogenomics in Therapeutics*. CRC Press.

123. Fenyvesi, F., Fenyvesi, É., Szente, L., Goda, K., Bacsó, Z., Bácskay, I., Váradi, J., Kiss, T., Molnár, É., Janáky, T., Szabó, G., & Vecsernyés, M. (2008). P-glycoprotein inhibition by membrane cholesterol modulation. *European Journal of Pharmaceutical Sciences*, 34(4), 236–242. <https://doi.org/10.1016/j.ejps.2008.04.005>

124. Ferguson, P. J., & Cass, C. E. (1985). Differential Cellular Retention of Vincristine and Vinblastine by Cultured Human Promyelocytic Leukemia HL-60/Cl Cells: The Basis of Differential Toxicity. *Cancer Research*, 45(11 Part 1), 5480–5488.

125. Finch, A., & Pillans, P. (2014). P-glycoprotein and its role in drug-drug interactions. *Australian Prescriber*, 37(4), 137–139. <https://doi.org/10.18773/austprescr.2014.050>

126. Foley, S. E., Tuohy, C., Dunford, M., Grey, M. J., De Luca, H., Cawley, C., Szabady, R. L., Maldonado-Contreras, A., Houghton, J. M., Ward, D. V., Mrsny, R. J., & McCormick, B. A. (2021). Gut microbiota regulation of P-glycoprotein in the intestinal epithelium in maintenance of homeostasis. *Microbiome*, 9(1), 183. <https://doi.org/10.1186/s40168-021-01137-3>

127. Foucaud-Vignault, M., Soayfane, Z., Ménez, C., Bertrand-Michel, J., Martin, P. G. P., Guillou, H., Collet, X., & Lespine, A. (2011). P-glycoprotein Dysfunction Contributes to Hepatic Steatosis and Obesity in Mice. *PLOS ONE*, 6(9), e23614. <https://doi.org/10.1371/journal.pone.0023614>

128. Frank, G. A., Shukla, S., Rao, P., Borgnia, M. J., Bartesaghi, A., Merk, A., Mobin, A., Esser, L., Earl, L. A., Gottesman, M. M., Xia, D., Ambudkar, S. V., & Subramaniam, S. (2016). Cryo-EM analysis of the conformational landscape of human P-glycoprotein (ABCB1) during its catalytic cycle. *Mol Pharmacol*, 90, 35–41. <https://doi.org/10.1124/mol.116.104190>
129. Fromm, M. F. (2004). Importance of P-glycoprotein at blood–tissue barriers. *Trends in Pharmacological Sciences*, 25(8), 423–429. <https://doi.org/10.1016/j.tips.2004.06.002>
130. Fu, D. (2013). Where is it and how does it get there – intracellular localization and traffic of P-glycoprotein. *Frontiers in Oncology*, 3, 1–5. <https://doi.org/10.3389/fonc.2013.00321>
131. Fu, D., & Roufogalis, B. D. (2007). Actin disruption inhibits endosomal traffic of P-glycoprotein-EGFP and resistance to daunorubicin accumulation. *American Journal of Physiology-Cell Physiology*, 292(4), C1543–C1552. <https://doi.org/10.1152/ajpcell.00068.2006>
132. Fujioka, S., Li, J., Choi, Y. H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., & Sakurai, A. (1997). The Arabidopsis deetiolated2 mutant is blocked early in brassinosteroid biosynthesis. *The Plant Cell*, 9(11), 1951–1962. <https://doi.org/10.1105/tpc.9.11.1951>
133. Fung, K. L., Pan, J., Ohnuma, S., Lund, P. E., Pixley, J. N., Kimchi-Sarfaty, C., Ambudkar, S. V., & Gottesman, M. M. (2014). MDR1 Synonymous Polymorphisms Alter Transporter Specificity and Protein Stability in a Stable Epithelial Monolayer. *Cancer Research*, 74(2), 598–608. <https://doi.org/10.1158/0008-5472.CAN-13-2064>
134. Futamata, R., Ogasawara, F., Ichikawa, T., Kodan, A., Kimura, Y., Kioka, N., & Ueda, K. (2020). In vivo FRET analyses reveal a role of ATP hydrolysis–associated conformational changes in human P-glycoprotein. *Journal of Biological Chemistry*, 295(15), 5002–5011. <https://doi.org/10.1074/jbc.RA119.012042>

135. Fyfe, P. K., McAuley, K. E., Roszak, A. W., Isaacs, N. W., Cogdell, R. J., & Jones, M. R. (2001). Probing the interface between membrane proteins and membrane lipids by X-ray crystallography. *Trends in Biochemical Sciences*, 26, 106–112.
136. Garrigos, M., Mir, L. M., & Orlowski, S. (1997). Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase—Further experimental evidence for a multisite model. *Eur J Biochem*, 244(2), 664–673.
137. Garrigues, A., Escargueil, A. E., & Orlowski, S. (2002). The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane. *Proc Natl Acad Sci U S A*, 99(16), 10347–10352. <https://doi.org/10.1073/pnas.162366399>
138. Gayer, A. V., Yakimov, B. P., Budylin, G. S., & Shirshin, E. A. (2020). Evaluating the Number of Ligand Binding Sites on Protein from Tryptophan Fluorescence Quenching under Typical Experimental Conditions. *Journal of Biomedical Photonics & Engineering*, 6(2), Article 2. <https://doi.org/10.18287/JBPE20.06.020303>
139. Gayet, L., Dayan, G., Barakat, S., Labialle, S., Michaud, M., Cogne, S., Mazane, A., Coleman, A. W., Rigal, D., & Baggetto, L. G. (2005). Control of P-Glycoprotein Activity by Membrane Cholesterol Amounts and Their Relation to Multidrug Resistance in Human CEM Leukemia Cells. *Biochemistry*, 44(11), 4499–4509. <https://doi.org/10.1021/bi048669w>
140. Geisler, M., & Murphy, A. S. (2006). The ABC of auxin transport: The role of p-glycoproteins in plant development. *FEBS Letters*, 580(4), 1094–1102. <https://doi.org/10.1016/j.febslet.2005.11.054>
141. Genovese, I., Ilari, A., Assaraf, Y. G., Fazi, F., & Colotti, G. (2017). Not only P-glycoprotein: Amplification of the ABCB1-containing chromosome region 7q21 confers multidrug

resistance upon cancer cells by coordinated overexpression of an assortment of resistance-related proteins. *Drug Resistance Updates*, 32, 23–46. <https://doi.org/10.1016/j.drug.2017.10.003>

142. Georgantzopoulou, A., Skoczylńska, E., Van den Berg, J. H. J., Brand, W., Legay, S., Klein, S. G., Rietjens, I. M. C. M., & Murk, A. J. (2014). P-gp efflux pump inhibition potential of common environmental contaminants determined in vitro. *Environmental Toxicology and Chemistry*, 33(4), 804–813. <https://doi.org/10.1002/etc.2493>

143. Gibbs, M. E., Wilt, L. A., Ledwitch, K. V., & Roberts, A. G. (2018). A conformationally-gated model of methadone and loperamide transport by P-glycoprotein. *Journal of Pharmaceutical Sciences*. <https://doi.org/10.1016/j.xphs.2018.02.019>

144. Giuseppina, G., Mariella, C., Mario, F. T., & Anna, C. (2011). Efficacy and Tolerability of Vinorelbine in the Cancer Therapy. *Current Drug Safety*, 6(3), 185–193.

145. Grube, M., Reuther, S., Schwabedissen, H. M. zu, Köck, K., Draber, K., Ritter, C. A., Fusch, C., Jedlitschky, G., & Kroemer, H. K. (2007). Organic Anion Transporting Polypeptide 2B1 and Breast Cancer Resistance Protein Interact in the Transepithelial Transport of Steroid Sulfates in Human Placenta. *Drug Metabolism and Disposition*, 35(1), 30–35. <https://doi.org/10.1124/dmd.106.011411>

146. Háda, V., Dubrovay, Z., Lakó-Futó, Á., Galambos, J., Gulyás, Z., Aranyi, A., & Szántay, C. (2013). NMR and mass spectrometric characterization of vinblastine, vincristine and some new related impurities—Part II. *Journal of Pharmaceutical and Biomedical Analysis*, 84, 309–322. <https://doi.org/10.1016/j.jpba.2012.09.008>

147. Han, E. S., & Zhang, J.-T. (1999). Membrane orientation of carboxyl-terminal half P-glycoprotein: Topogenesis of transmembrane segments. *European Journal of Cell Biology*, 78(9), 624–631. [https://doi.org/10.1016/S0171-9335\(99\)80047-8](https://doi.org/10.1016/S0171-9335(99)80047-8)

148. Haselhorst, T., Munster-Kuhnel, A. K., Oeschles, M., Tiralongo, J., Gerardy-Schahn, R., & von Itzstein, M. (2007). Direct detection of ligand binding to Sepharose-immobilised protein using saturation transfer double difference (STDD) NMR spectroscopy. *Biochemical and Biophysical Research Communications*, 359, 866–870. <https://doi.org/10.1016/j.bbrc.2007.05.204>
149. Hau, R. K., Wright, S. H., & Cherrington, N. J. (2023). Drug Transporters at the Human Blood-Testis Barrier. *Drug Metabolism and Disposition*, 51(5), 560–571. <https://doi.org/10.1124/dmd.122.001186>
150. Hecht, S. M. (2005). Camptothecin: Roles of the D and E rings in binding to the topoisomerase I-DNA covalent binary complex. *Current Medicinal Chemistry. Anti-Cancer Agents*, 5(4), 353–362. <https://doi.org/10.2174/1568011054222373>
151. Hennessy, M., & Spiers, J. P. (2007). A primer on the mechanics of P-glycoprotein the multidrug transporter. *Pharmacological Research*, 55(1), 1–15. <https://doi.org/10.1016/j.phrs.2006.10.007>
152. Henrique, R., Oliveira, A. I., Costa, V. L., Baptista, T., Martins, A. T., Morais, A., Oliveira, J., & Jerónimo, C. (2013). Epigenetic regulation of MDR1 gene through post-translational histone modifications in prostate cancer. *BMC Genomics*, 14(1), 898. <https://doi.org/10.1186/1471-2164-14-898>
153. Hirose, K., Kozu, C., Yamashita, K., Maruo, E., Kitamura, M., Hasegawa, J., Omoda, K., Murakami, T., & Maeda, Y. (2012). Correlation between plasma concentration ratios of SN-38 glucuronide and SN-38 and neutropenia induction in patients with colorectal cancer and wild-type UGT1A1 gene. *Oncology Letters*, 3(3), 694–698. <https://doi.org/10.3892/ol.2011.533>
154. Hoosain, F. G., Choonara, Y. E., Tomar, L. K., Kumar, P., Tyagi, C., du Toit, L. C., & Pillay, V. (2015). Bypassing P-Glycoprotein Drug Efflux Mechanisms: Possible Applications in

Pharmacoresistant Schizophrenia Therapy. *BioMed Research International*, 2015.
<https://doi.org/10.1155/2015/484963>

155. Houghton, P. J., Cheshire, P. J., Hallman, J. D., Lutz, L., Friedman, H. S., Danks, M. K., & Houghton, J. A. (1995). Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemotherapy and Pharmacology*, 36(5), 393–403.
<https://doi.org/10.1007/BF00686188>

156. Hsin, C., Stoffel, M. S., Gazzaz, M., Schaeffeler, E., Schwab, M., Fuhr, U., & Taubert, M. (2020). Combinations of common SNPs of the transporter gene ABCB1 influence apparent bioavailability, but not renal elimination of oral digoxin. *Scientific Reports*, 10, 12457.
<https://doi.org/10.1038/s41598-020-69326-y>

157. Husain, A., Makadia, V., Valicherla, G. R., Riyazuddin, M., & Gayen, J. R. (2022). Approaches to minimize the effects of P-glycoprotein in drug transport: A review. *Drug Development Research*, 83(4), 825–841. <https://doi.org/10.1002/ddr.21918>

158. Hyz, K., Kawęcki, R., Bednarek, E., Bocian, W., Sitkowski, J., & Kozerski, L. (2010). Topotecan dynamics, tautomerism and reactivity—¹H/¹³C NMR and ESI MS study. *Magnetic Resonance in Chemistry*, 48(8), 575–584. <https://doi.org/10.1002/mrc.2625>

159. Iqbal, S., Flux, C., Briggs, D. A., Deplazes, E., Long, J., Skrzypek, R., Rothnie, A., Kerr, I. D., & Callaghan, R. (2022). Vinca alkaloid binding to P-glycoprotein occurs in a processive manner. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1864(10), 184005.
<https://doi.org/10.1016/j.bbamem.2022.184005>

160. Jain, R., Majumdar, S., Nashed, Y., Pal, D., & Mitra, A. K. (2004). Circumventing P-Glycoprotein-Mediated Cellular Efflux of Quinidine by Prodrug Derivatization. *Molecular Pharmaceutics*, 1(4), 290–299. <https://doi.org/10.1021/mp049952s>
161. Jasiński, M., Stukkens, Y., Degand, H., Purnelle, B., Marchand-Brynaert, J., & Boutry, M. (2001). A Plant Plasma Membrane ATP Binding Cassette-Type Transporter Is Involved in Antifungal Terpenoid Secretion. *The Plant Cell*, 13(5), 1095–1108.
162. Jazaj, D., Ghadami, S. A., Bemporad, F., & Chiti, F. (2019). Probing conformational changes of monomeric transthyretin with second derivative fluorescence. *Scientific Reports*, 9(1), Article 1. <https://doi.org/10.1038/s41598-019-47230-4>
163. Jeewantha, H. A., Ivanovich, S. A., & Mihailovich, K. P. (2017). VALIDATED SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF VINCRIStINE AND VINBLASTINE. *International Journal of Pharmacy and Pharmaceutical Sciences*, 78–86. <https://doi.org/10.22159/ijpps.2017v9i4.16577>
164. Jin, M. S., Oldham, M. L., Zhang, Q., & Chen, J. (2012). Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. *Nature*, 490, 566–569. <https://doi.org/10.1038/nature11448>
165. Jing, H., Wang, Y., Desai, P. R., Ramamurthi, K. S., & Das, S. (2020). Lipid flip-flop and desorption from supported lipid bilayers is independent of curvature. *PLoS ONE*, 15(12), e0244460. <https://doi.org/10.1371/journal.pone.0244460>
166. Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H., & Shu, Y. (2019). Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Molecular Cancer*, 18(1), 157. <https://doi.org/10.1186/s12943-019-1089-9>

167. Joto, N., Ishii, M., Minami, M., Kuga, H., Mitsui, I., & Tohgo, A. (1997). DX-8951F, a water-soluble camptothecin analog, exhibits potent antitumor activity against a human lung cancer cell line and its SN-38-resistant variant. *International Journal of Cancer*, 72(4), 680–686. [https://doi.org/10.1002/\(SICI\)1097-0215\(19970807\)72:4<680::AID-IJC21>3.0.CO;2-E](https://doi.org/10.1002/(SICI)1097-0215(19970807)72:4<680::AID-IJC21>3.0.CO;2-E)
168. Juliano, R. L., & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 455(1), 152–162. [https://doi.org/10.1016/0005-2736\(76\)90160-7](https://doi.org/10.1016/0005-2736(76)90160-7)
169. Kacprzak, K. M. (2013). Chemistry and Biology of Camptothecin and its Derivatives. In K. G. Ramawat & J.-M. Mérillon (Eds.), *Natural Products* (pp. 643–682). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-22144-6_26
170. Kamau, S. W., Krämer, S. D., Günthert, M., & Wunderli-Allenspach, H. (2005). Effect of the modulation of the membrane lipid composition on the localization and function of P-glycoprotein in MDR1-MDCK cells. *In Vitro Cellular & Developmental Biology - Animal*, 41(7), 207–216. <https://doi.org/10.1290/0502016.1>
171. Kapse-Mistry, S., Govender, T., Srivastava, R., & Yergeri, M. (2014). Nanodrug delivery in reversing multidrug resistance in cancer cells. *Frontiers in Pharmacology*, 5. <https://www.frontiersin.org/articles/10.3389/fphar.2014.00159>
172. Karthika, C., & Sureshkumar, R. (2020). P-Glycoprotein Efflux Transporters and Its Resistance Its Inhibitors and Therapeutic Aspects. In *Biomarkers and Bioanalysis Overview*. IntechOpen. <https://doi.org/10.5772/intechopen.90430>
173. Karthika, C., Sureshkumar, R., Zehravi, M., Akter, R., Ali, F., Ramproshad, S., Mondal, B., Tagde, P., Ahmed, Z., Khan, F. S., Rahman, M. H., & Cavalu, S. (2022). Multidrug Resistance

of Cancer Cells and the Vital Role of P-Glycoprotein. *Life*, 12(6), Article 6.
<https://doi.org/10.3390/life12060897>

174. Katayama, K., Noguchi, K., & Sugimoto, Y. (2014). Regulations of P-Glycoprotein/ABCB1/MDR1 in Human Cancer Cells. *New Journal of Science*.
<https://doi.org/10.1155/2014/476974>

175. Kawahara, I., Nishikawa, S., Yamamoto, A., Kono, Y., & Fujita, T. (2020). The Impact of Breast Cancer Resistance Protein (BCRP/ABCG2) on Drug Transport Across Caco-2 Cell Monolayers. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, 48(6), 491–498. <https://doi.org/10.1124/dmd.119.088674>

176. Kciuk, M., Marciniak, B., & Kontek, R. (2020). Irinotecan—Still an Important Player in Cancer Chemotherapy: A Comprehensive Overview. *International Journal of Molecular Sciences*, 21(14), Article 14. <https://doi.org/10.3390/ijms21144919>

177. Keogh, J. P., & Kunta, J. R. (2006). Development, validation and utility of an in vitro technique for assessment of potential clinical drug-drug interactions involving P-glycoprotein. *European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences*, 27(5), 543–554. <https://doi.org/10.1016/j.ejps.2005.11.011>

178. Kerr, K. M., Sauna, Z. E., & Ambudkar, S. V. (2001). Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in Human P-glycoprotein. Evidence for ADP release as the rate-limiting step in the catalytic cycle and its modulation by substrates. *J Biol Chem*, 276(12), 8657–8664. <https://doi.org/10.1074/jbc.M010044200>

179. Kiela, P. R., & Ghishan, F. K. (2016). Physiology of Intestinal Absorption and Secretion. *Best Practice & Research. Clinical Gastroenterology*, 30(2), 145–159.
<https://doi.org/10.1016/j.bpg.2016.02.007>

180. Kikuchi, R., de Morais, S. M., & Kalvass, J. C. (2013). In vitro P-glycoprotein efflux ratio can predict the in vivo brain penetration regardless of biopharmaceutics drug disposition classification system class. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, 41, 2012–2017. <https://doi.org/10.1124/dmd.113.053868>
181. Kim, Y., & Chen, J. (2018). Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. *Science*, 359(6378), 915–919. <https://doi.org/10.1126/science.aar7389>
182. Kimura, Y., Kioka, N., Kato, H., Matsuo, M., & Ueda, K. (2007). Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. *Biochem J*, 401(2), 597–605. <https://doi.org/10.1042/BJ20060632>
183. Kipp, H., & Arias, I. M. (2000). Newly Synthesized Canalicular ABC Transporters Are Directly Targeted from the Golgi to the Hepatocyte Apical Domain in Rat Liver*. *Journal of Biological Chemistry*, 275(21), 15917–15925. <https://doi.org/10.1074/jbc.M909875199>
184. Kodan, A., Shibata, H., Matsumoto, T., Terakado, K., Sakiyama, K., Matsuo, M., Ueda, K., & Kato, H. (2009). Improved expression and purification of human multidrug resistance protein MDR1 from baculovirus-infected insect cells. *Protein Expression and Purification*, 66(1), 7–14. <https://doi.org/10.1016/j.pep.2009.02.010>
185. Kodan, A., Yamaguchi, T., Nakatsu, T., Matsuoka, K., Kimura, Y., Ueda, K., & Kato, H. (2019). Inward- and outward-facing X-ray crystal structures of homodimeric P-glycoprotein CmABCB1. *Nature Communications*, 10(1), Article 1. <https://doi.org/10.1038/s41467-018-08007->

186. Kondrashov, D. A., Van Wynsberghe, A. W., Bannen, R. M., Cui, Q., & Phillips, G. N. (2007). Protein Structural Variation in Computational Models and Crystallographic Data. *Structure*, 15(2), 169–177. <https://doi.org/10.1016/j.str.2006.12.006>
187. Kong, L.-L., Shen, G.-L., Wang, Z.-Y., Zhuang, X.-M., Xiao, W.-B., Yuan, M., Gong, Z.-H., & Li, H. (2016). Inhibition of P-glycoprotein and multidrug resistance-associated protein 2 regulates the hepatobiliary excretion and plasma exposure of thienorphine and Its glucuronide conjugate. *Frontiers in Pharmacology*, 7. <https://doi.org/10.3389/fphar.2016.00242>
188. Kooij, G., Backer, R., Koning, J. J., Reijerkerk, A., Horssen, J. van, Pol, S. M. A. van der, Drexhage, J., Schinkel, A., Dijkstra, C. D., Haan, J. M. M. den, Geijtenbeek, T. B. H., & Vries, H. E. de. (2009). P-Glycoprotein Acts as an Immunomodulator during Neuroinflammation. *PLOS ONE*, 4(12), e8212. <https://doi.org/10.1371/journal.pone.0008212>
189. Kostjukov, V. V. (2021). Theoretical analysis of lactone and carboxylate forms of camptothecin in aqueous solution: Electronic states, absorption spectra, and hydration. *Journal of Molecular Liquids*, 344, 117804. <https://doi.org/10.1016/j.molliq.2021.117804>
190. Krikorian, A., & Breillout, F. (1991). Vinorelbine (Navelbine). A new semisynthetic vinca alkaloid. *Onkologie*, 14(1), 7–12. <https://doi.org/10.1159/000216938>
191. Kruczynski, A., Barret, J.-M., Etiévant, C., Colpaert, F., Fahy, J., & Hill, B. T. (1998). Antimitotic and tubulin-interacting properties of vinflunine, a novel fluorinated Vinca alkaloid. *Biochemical Pharmacology*, 55(5), 635–648. [https://doi.org/10.1016/S0006-2952\(97\)00505-4](https://doi.org/10.1016/S0006-2952(97)00505-4)
192. Kruczynski, A., & Hill, B. T. (2001). Vinflunine, the latest Vinca alkaloid in clinical development: A review of its preclinical anticancer properties. *Critical Reviews in Oncology/Hematology*, 40(2), 159–173. [https://doi.org/10.1016/S1040-8428\(01\)00183-4](https://doi.org/10.1016/S1040-8428(01)00183-4)

193. Kruh, G. D., & Belinsky, M. G. (2003). The MRP family of drug efflux pumps. *Oncogene*, 22(47), 7537–7552. <https://doi.org/10.1038/sj.onc.1206953>
194. Kulig, W., Olżyńska, A., Jurkiewicz, P., Kantola, A. M., Komulainen, S., Manna, M., Pourmousa, M., Vazdar, M., Cwiklik, L., Rog, T., Khelashvili, G., Harries, D., Telkki, V.-V., Hof, M., Vattulainen, I., & Jungwirth, P. (2015). Cholesterol under oxidative stress—How lipid membranes sense oxidation as cholesterol is being replaced by oxysterols. *Free Radical Biology and Medicine*, 84, 30–41. <https://doi.org/10.1016/j.freeradbiomed.2015.03.006>
195. Kumar, A., Patil, D., Rajamohanan, P. R., & Ahmad, A. (2013). Isolation, Purification and Characterization of Vinblastine and Vincristine from Endophytic Fungus *Fusarium oxysporum* Isolated from *Catharanthus roseus*. *PLOS ONE*, 8(9), e71805. <https://doi.org/10.1371/journal.pone.0071805>
196. Kuzu, O. F., Noory, M. A., & Robertson, G. P. (2016). The role of cholesterol in cancer. *Cancer Research*, 76(8), 2063–2070. <https://doi.org/10.1158/0008-5472.CAN-15-2613>
197. Kwan, P., Baum, L., Wong, V., Ng, P. W., Lui, C. H., Sin, N. C., Hui, A. C. F., Yu, E., & Wong, L. K. S. (2007). Association between ABCB1 C3435T polymorphism and drug-resistant epilepsy in Han Chinese. *Epilepsy & Behavior*, 11(1), 112–117. <https://doi.org/10.1016/j.yebeh.2007.04.013>
198. Laco, G. S. (2011). Evaluation of Two Models for Human Topoisomerase I Interaction with dsDNA and Camptothecin Derivatives. *PLOS ONE*, 6(8), e24314. <https://doi.org/10.1371/journal.pone.0024314>
199. Lai, J.-I., Tseng, Y.-J., Chen, M.-H., Huang, C.-Y. F., & Chang, P. M.-H. (2020). Clinical Perspective of FDA Approved Drugs With P-Glycoprotein Inhibition Activities for Potential

<https://www.frontiersin.org/articles/10.3389/fonc.2020.561936>

200. Lakowicz, J. R. (2011). *Principles of Fluorescence Spectroscopy* (3rd edition). Springer.
201. Lam, J., Baello, S., Iqbal, M., Kelly, L. E., Shannon, P. T., Chitayat, D., Matthews, S. G., & Koren, G. (2015). The ontogeny of P-glycoprotein in the developing human blood–brain barrier: Implication for opioid toxicity in neonates. *Pediatric Research*, 78(4), Article 4. <https://doi.org/10.1038/pr.2015.119>
202. Lankas, G. R., Cartwright, M. E., & Umbenhauer, D. (1997). P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicology and Applied Pharmacology*, 143(2), 357–365. <https://doi.org/10.1006/taap.1996.8086>
203. Lankas, G. R., Wise, L. D., Cartwright, M. E., Pippert, T., & Umbenhauer, D. R. (1998). Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reproductive Toxicology (Elmsford, N.Y.)*, 12(4), 457–463. [https://doi.org/10.1016/S0890-6238\(98\)00027-6](https://doi.org/10.1016/S0890-6238(98)00027-6)
204. Lea-Henry, T. N., Carland, J. E., Stocker, S. L., Sevastos, J., & Roberts, D. M. (2018). Clinical Pharmacokinetics in Kidney Disease: Fundamental Principles. *Clinical Journal of the American Society of Nephrology*, 13(7), 1085. <https://doi.org/10.2215/CJN.00340118>
205. Ledwitch, K. V., Barnes, R. W., & Roberts, A. G. (2016). Unravelling the complex drug-drug interactions of the cardiovascular drugs, verapamil and digoxin, with P-glycoprotein. *Bioscience Reports*, 36(2), 1–14. <https://doi.org/10.1042/BSR20150317>
206. Ledwitch, K. V., Gibbs, M. E., Barnes, R. W., & Roberts, A. G. (2016). Cooperativity between verapamil and ATP bound to the efflux transporter P-glycoprotein. *Biochemical Pharmacology*, 118, 96–108. <https://doi.org/10.1016/j.bcp.2016.08.013>

207. Lee, J., Kang, J., Kwon, N.-Y., Sivaraman, A., Naik, R., Jin, S.-Y., Oh, A. R., Shin, J.-H., Na, Y., Lee, K., & Lee, H.-J. (2021). Dual Inhibition of P-gp and BCRP Improves Oral Topotecan Bioavailability in Rodents. *Pharmaceutics*, 13(4), 559. <https://doi.org/10.3390/pharmaceutics13040559>
208. Leeson, P. D., & Springthorpe, B. (2007). The influence of drug-like concepts on decision-making in medicinal chemistry. *Nature Reviews Drug Discovery*, 6(11), 881–890. <https://doi.org/10.1038/nrd2445>
209. Lerner-Marmarosh, N., Gimi, K., Urbatsch, I. L., Gros, P., & Senior, A. E. (1999). Large scale purification of detergent-soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding properties of wild-type, walker A, and walker B mutant proteins. *Journal of Biological Chemistry*, 274, 34711–34718. <https://doi.org/10.1074/jbc.274.49.34711>
210. Leslie, E. M., Deeley, R. G., & Cole, S. P. (2005). Multidrug resistance proteins: Role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicology and Applied Pharmacology*, 204, 216–237.
211. Li, F., Jiang, T., Li, Q., & Ling, X. (2017). Camptothecin (CPT) and its derivatives are known to target topoisomerase I (Top1) as their mechanism of action: Did we miss something in CPT analogue molecular targets for treating human disease such as cancer? *American Journal of Cancer Research*, 7(12), 2350–2394.
212. Li, H., Jin, H.-E., Kim, W., Han, Y.-H., Kim, D.-D., Chung, S.-J., & Shim, C.-K. (2008). Involvement of P-glycoprotein, multidrug resistance protein 2 and breast cancer resistance protein in the transport of belotecan and topotecan in Caco-2 and MDCKII cells. *Pharmaceutical Research*, 25(11), 2601–2612. <https://doi.org/10.1007/s11095-008-9678-0>

213. Li, J., Jaimes, K. F., & Aller, S. G. (2014). Refined structures of mouse P-glycoprotein. *Protein Sci*, 23, 34–46. <https://doi.org/10.1002/pro.2387>
214. Li, L.-C., Carroll, P. R., & Dahiya, R. (2005). Epigenetic Changes in Prostate Cancer: Implication for Diagnosis and Treatment. *JNCI: Journal of the National Cancer Institute*, 97(2), 103–115. <https://doi.org/10.1093/jnci/dji010>
215. Li, M., Dang, D., Liu, L., Xi, N., & Wang, Y. (2017). Imaging and Force Recognition of Single Molecular Behaviors Using Atomic Force Microscopy. *Sensors*, 17(1), Article 1. <https://doi.org/10.3390/s17010200>
216. Li, R., Maurer, T. S., Sweeney, K., & Barton, H. A. (2016). Does the Systemic Plasma Profile Inform the Liver Profile? Analysis Using a Physiologically Based Pharmacokinetic Model and Individual Compounds. *The AAPS Journal*, 18(3), 746–756. <https://doi.org/10.1208/s12248-016-9895-0>
217. Li, Y., Yuan, H., Yang, K., Xu, W., Tang, W., & Li, X. (2010). The structure and functions of P-glycoprotein. *Current Medicinal Chemistry*, 17(8), 786–800. <https://doi.org/10.2174/092986710790514507>
218. Li, Y.-H., Wang, Y.-H., Li, Y., & Yang, L. (2006). MDR1 Gene Polymorphisms and Clinical Relevance. *Acta Genetica Sinica*, 33(2), 93–104. [https://doi.org/10.1016/S0379-4172\(06\)60027-9](https://doi.org/10.1016/S0379-4172(06)60027-9)
219. Liang, Y., Li, S., & Chen, L. (2015). The physiological role of drug transporters. *Protein & Cell*, 6(5), 334–350. <https://doi.org/10.1007/s13238-015-0148-2>
220. Li-Blatter, X., Beck, A., & Seelig, A. (2012). P-Glycoprotein-ATPase Modulation: The Molecular Mechanisms. *Biophysical Journal*, 102(6), 1383–1393. <https://doi.org/10.1016/j.bpj.2012.02.018>

221. Li-Blatter, X., Nervi, P., & Seelig, A. (2009). Detergents as intrinsic P-glycoprotein substrates and inhibitors. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1788(10), 2335–2344. <https://doi.org/10.1016/j.bbamem.2009.07.010>
222. Lin, X., Skolnik, S., Chen, X., & Wang, J. (2011). Attenuation of Intestinal Absorption by Major Efflux Transporters: Quantitative Tools and Strategies Using a Caco-2 Model. *Drug Metabolism and Disposition*, 39(2), 265–274. <https://doi.org/10.1124/dmd.110.034629>
223. Linn, S. C., & Giaccone, G. (1995). MDR1/P-glycoprotein expression in colorectal cancer. *European Journal of Cancer*, 31(7), 1291–1294. [https://doi.org/10.1016/0959-8049\(95\)00278-Q](https://doi.org/10.1016/0959-8049(95)00278-Q)
224. Linn, S. C., Honkoop, A. H., Hoekman, K., van der Valk, P., Pinedo, H. M., & Giaccone, G. (1996). P53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *British Journal of Cancer*, 74(1), Article 1. <https://doi.org/10.1038/bjc.1996.316>
225. Linton, K. J., & Higgins, C. F. (1998). The Escherichia coli ATP-binding cassette (ABC) proteins. *Molecular Microbiology*, 28(1), 5–13. <https://doi.org/10.1046/j.1365-2958.1998.00764.x>
226. Lipinski, C. A. (2004). Lead-and drug-like compounds: The rule-of-five revolution. *Drug Discovery Today: Technologies*, 1, 337–341.
227. Litman, T., Zeuthen, T., Skovsgaard, T., & Stein, W. D. (1997). Structure-activity relationships of P-glycoprotein interacting drugs: Kinetic characterization of their effects on ATPase activity. *Biochim Biophys Acta*, 1361(2), 159–168.
228. Liu, J., Liu, Y., Powell, D. A., Waalkes, M. P., & Klaassen, C. D. (2002). Multidrug-resistance mdr1a/1b double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology*, 170(1), 55–62. [https://doi.org/10.1016/S0300-483X\(01\)00532-7](https://doi.org/10.1016/S0300-483X(01)00532-7)

229. Liu, M. L., Mao, X. A., Ye, C. H., Huang, H., Nicholson, J. K., & Lindon, J. C. (1998). Improved WATERGATE pulse sequences for solvent suppression in NMR spectroscopy. *J Magn Reson*, 132, 125–129. <https://doi.org/DOI 10.1006/jmre.1998.1405>
230. Liu, R., Chen, Y., Liu, G., Li, C., Song, Y., Cao, Z., Li, W., Hu, J., Lu, C., & Liu, Y. (2020). PI3K/AKT pathway as a key link modulates the multidrug resistance of cancers. *Cell Death & Disease*, 11(9), 797. <https://doi.org/10.1038/s41419-020-02998-6>
231. Liu, R., Siemiarczuk, A., & Sharom, F. J. (2000). Intrinsic fluorescence of the P-glycoprotein multidrug transporter: Sensitivity of tryptophan residues to binding of drugs and nucleotides. *Biochemistry*, 39, 14927–14938. <https://doi.org/10.1021/bi0018786>
232. Lobert, S., Vulevic, B., & Correia, J. J. (1996). Interaction of vinca alkaloids with tubulin: A comparison of vinblastine, vincristine, and vinorelbine. *Biochemistry*, 35(21), 6806–6814. <https://doi.org/10.1021/bi953037i>
233. Longley, D. B., & Johnston, P. G. (2005). Molecular mechanisms of drug resistance. *The Journal of Pathology*, 205(2), 275–292. <https://doi.org/10.1002/path.1706>
234. Loo, T. W., Bartlett, M. C., & Clarke, D. M. (2003). Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains. *Journal of Biological Chemistry*, 278, 1575–1578. <https://doi.org/10.1074/jbc.M211307200>
235. Loo, T. W., Bartlett, M. C., & Clarke, D. M. (2006). Transmembrane segment 7 of human P-glycoprotein forms part of the drug-binding pocket. *Biochem J*, 399(2), 351–359. <https://doi.org/10.1042/BJ20060715>
236. Loo, T. W., Bartlett, M. C., & Clarke, D. M. (2010). Human P-glycoprotein is active when the two halves are clamped together in the closed conformation. *Biochemical and Biophysical Research Communications*, 395, 436–440.

237. Loo, T. W., Bartlett, M. C., Detty, M. R., & Clarke, D. M. (2012). The ATPase activity of the P-glycoprotein drug pump is highly activated when the N-terminal and central regions of the nucleotide-binding domains are linked closely together. *Journal of Biological Chemistry*, 287, 26806–26816.
238. Loo, T. W., & Clarke, D. M. (1995). Membrane topology of a cysteine-less mutant of human p-glycoprotein. *Journal of Biological Chemistry*, 270, 843–848.
239. Loo, T. W., & Clarke, D. M. (2014a). Cysteines introduced into extracellular loops 1 and 4 of human P-glycoprotein that are close only in the open conformation spontaneously form a disulfide bond that inhibits drug efflux and ATPase activity. *Journal of Biological Chemistry*, 289, 24749–24758. <https://doi.org/10.1074/jbc.M114.583021>
240. Loo, T. W., & Clarke, D. M. (2014b). Locking intracellular helices 2 and 3 together inactivates human P-glycoprotein. *Journal of Biological Chemistry*, 289, 229–236.
241. Loo, T. W., & Clarke, D. M. (2014c). Tariquidar inhibits P-glycoprotein drug efflux but activates ATPase activity by blocking transition to an open conformation. *Biochemical Pharmacology*, 92(4), 558–566. <https://doi.org/10.1016/j.bcp.2014.10.006>
242. Loo, T. W., & Clarke, D. M. (2015). The transmission interfaces contribute asymmetrically to the assembly and activity of human P-glycoprotein. *Journal of Biological Chemistry*, 290, 16954–16963.
243. Loo, T. W., & Clarke, D. M. (2016). P-glycoprotein ATPase activity requires lipids to activate a switch at the first transmission interface. *Biochemical and Biophysical Research Communications*, 472(2), 379–383. <https://doi.org/10.1016/j.bbrc.2016.02.124>

244. Löscher, W., & Gericke, B. (2020). Novel Intrinsic Mechanisms of Active Drug Extrusion at the Blood-Brain Barrier: Potential Targets for Enhancing Drug Delivery to the Brain? *Pharmaceutics*, 12(10). <https://doi.org/10.3390/pharmaceutics12100966>
245. Luker, G. D., Pica, C. M., Kumar, A. S., Covey, D. F., & Piwnica-Worms, D. (2000). Effects of Cholesterol and Enantiomeric Cholesterol on P-Glycoprotein Localization and Function in Low-Density Membrane Domains. *Biochemistry*, 39(26), 7651–7661. <https://doi.org/10.1021/bi9928593>
246. Mahar Doan, K. M., Humphreys, J. E., Webster, L. O., Wring, S. A., Shampine, L. J., Serabjit-Singh, C. J., Adkison, K. K., & Polli, J. W. (2002). Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *Journal of Pharmacology and Experimental Therapeutics*, 303, 1029–1037. <https://doi.org/10.1124/jpet.102.039255>
247. Mai, Y., Dou, L., Yao, Z., Madla, C. M., Gavins, F. K. H., Taherali, F., Yin, H., Orlu, M., Murdan, S., & Basit, A. W. (2021). Quantification of P-Glycoprotein in the Gastrointestinal Tract of Humans and Rodents: Methodology, Gut Region, Sex, and Species Matter. *Molecular Pharmaceutics*, 18(5), 1895–1904. <https://doi.org/10.1021/acs.molpharmaceut.0c00574>
248. Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S., & Baradaran, B. (2017). The Different Mechanisms of Cancer Drug Resistance: A Brief Review. *Advanced Pharmaceutical Bulletin*, 7(3), 339–348. <https://doi.org/10.15171/apb.2017.041>
249. Mao, Q., Lai, Y., & Wang, J. (2018). Drug Transporters in Xenobiotic Disposition and Pharmacokinetic Prediction. *Drug Metabolism and Disposition*, 46(5), 561–566. <https://doi.org/10.1124/dmd.118.081356>

250. Marsh, B. P., Chada, N., Gari, R. R. S., Sigdel, K. P., & King, G. M. (2018). The hessian blob algorithm: Precise particle detection in atomic force microscopy imagery. *Scientific Reports*, 8(1), 978. <https://doi.org/10.1038/s41598-018-19379-x>
251. Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P., & Callaghan, R. (1999). The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. *British Journal of Pharmacology*, 128(2), 403–411. <https://doi.org/10.1038/sj.bjp.0702807>
252. Martin, C., Higgins, C. F., & Callaghan, R. (2001). The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. *Biochemistry*, 40(51), 15733–15742.
253. Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, Ü., Müller-Röber, B., & Schulz, B. (2002). Multifunctionality of plant ABC transporters—More than just detoxifiers. *Planta*, 214(3), 345–355.
254. Mayer, M., & Meyer, B. (2001). Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor. *J Am Chem Soc*, 123, 6108–6117.
255. Médioni, J., Di Palma, M., Guillot, A., Spaeth, D., & Théodore, C. (2016). Efficacy and safety of Vinflunine for advanced or metastatic urothelial carcinoma in routine practice based on the French multi-centre CURVE study. *BMC Cancer*, 16, 217. <https://doi.org/10.1186/s12885-016-2262-9>
256. Megli, C. J., & Coyne, C. B. (2022). Infections at the maternal–fetal interface: An overview of pathogenesis and defence. *Nature Reviews Microbiology*, 20(2), Article 2. <https://doi.org/10.1038/s41579-021-00610-y>

257. Melaine, N., Liénard, M.-O., Dorval, I., Le Goascogne, C., Lejeune, H., & Jégou, B. (2002). Multidrug Resistance Genes and P-Glycoprotein in the Testis of the Rat, Mouse, Guinea Pig, and Human. *Biology of Reproduction*, 67(6), 1699–1707. <https://doi.org/10.1095/biolreprod.102.003558>
258. Ménez, C., Mselli-Lakhal, L., Foucaud-Vignault, M., Balaguer, P., Alvinerie, M., & Lespine, A. (2012). Ivermectin induces P-glycoprotein expression and function through mRNA stabilization in murine hepatocyte cell line. *Biochemical Pharmacology*, 83(2), 269–278. <https://doi.org/10.1016/j.bcp.2011.10.010>
259. Mills, R. F., Francini, A., Ferreira da Rocha, P. S. C., Baccarini, P. J., Aylett, M., Krijger, G. C., & Williams, L. E. (2005). The plant P1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS Letters*, 579(3), 783–791. <https://doi.org/10.1016/j.febslet.2004.12.040>
260. Moeller, A., Lee, S. C., Tao, H., Speir, J. A., Chang, G., Urbatsch, I. L., Potter, C. S., Carragher, B., & Zhang, Q. (2015). Distinct conformational spectrum of homologous multidrug ABC transporters. *Structure (London, England : 1993)*, 23(3), 450–460. <https://doi.org/10.1016/j.str.2014.12.013>
261. Molinari, A., Cianfriglia, M., Meschini, S., Calcabrini, A., & Arancia, G. (1994). P-glycoprotein expression in the Golgi apparatus of multidrug-resistant cells. *International Journal of Cancer*, 59(6), 789–795. <https://doi.org/10.1002/ijc.2910590614>
262. Moller, M., & Denicola, A. (2002). Protein tryptophan accessibility studied by fluorescence quenching. *Biochemistry and Molecular Biology Education*, 30(3), 175–178. <https://doi.org/10.1002/Bmb.2002.494030030035>

263. Monaco, S., Tailford, L. E., Juge, N., & Angulo, J. (2017). Differential Epitope Mapping by STD NMR Spectroscopy To Reveal the Nature of Protein–Ligand Contacts. *Angewandte Chemie (International Ed. in English)*, 56(48), 15289–15293. <https://doi.org/10.1002/anie.201707682>
264. Moudi, M., Go, R., Yien, C. Y. S., & Nazre, Mohd. (2013). Vinca Alkaloids. *International Journal of Preventive Medicine*, 4(11), 1231–1235.
265. Mukherjee, S., & Mani, S. (2010). Orphan Nuclear Receptors as Targets for Drug Development. *Pharmaceutical Research*, 27(8), 1439–1468. <https://doi.org/10.1007/s11095-010-0117-7>
266. Müller, D. J., Dumitru, A. C., Lo Giudice, C., Gaub, H. E., Hinterdorfer, P., Hummer, G., De Yoreo, J. J., Dufrêne, Y. F., & Alsteens, D. (2021). Atomic Force Microscopy-Based Force Spectroscopy and Multiparametric Imaging of Biomolecular and Cellular Systems. *Chemical Reviews*, 121(19), 11701–11725. <https://doi.org/10.1021/acs.chemrev.0c00617>
267. Munteanu, E., Verdier, M., Grandjean-Forestier, F., Stenger, C., Jayat-Vignoles, C., Huet, S., Robert, J., & Ratinaud, M.-H. (2006). Mitochondrial localization and activity of P-glycoprotein in doxorubicin-resistant K562 cells. *Biochemical Pharmacology*, 71(8), 1162–1174. <https://doi.org/10.1016/j.bcp.2006.01.006>
268. Müsch, A., Cohen, D., & Rodriguez-Boulan, E. (1997). Myosin II Is Involved in the Production of Constitutive Transport Vesicles from the TGN. *Journal of Cell Biology*, 138(2), 291–306. <https://doi.org/10.1083/jcb.138.2.291>
269. Nabekura, T., Kamiyama, S., & Kitagawa, S. (2005). Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. *Biochemical and Biophysical Research Communications*, 327(3), 866–870. <https://doi.org/10.1016/j.bbrc.2004.12.081>

270. Nakao, H., Ikeda, K., Ishihama, Y., & Nakano, M. (2016). Membrane-Spanning Sequences in Endoplasmic Reticulum Proteins Promote Phospholipid Flip-Flop. *Biophysical Journal*, 110(12), 2689–2697. <https://doi.org/10.1016/j.bpj.2016.05.023>
271. Nanayakkara, A. K., Follit, C. A., Chen, G., Williams, N. S., Vogel, P. D., & Wise, J. G. (2018). Targeted inhibitors of P-glycoprotein increase chemotherapeutic-induced mortality of multidrug resistant tumor cells. *Scientific Reports*, 8(1), 967. <https://doi.org/10.1038/s41598-018-19325-x>
272. Nervi, P., Li-Blatter, X., Aanismaa, P., & Seelig, A. (2010). P-glycoprotein substrate transport assessed by comparing cellular and vesicular ATPase activity. *Biochim Biophys Acta*, 1798(3), 515–525. <https://doi.org/10.1016/j.bbamem.2009.11.022>
273. Ngan, V. K., Bellman, K., Panda, D., Hill, B. T., Jordan, M. A., & Wilson, L. (2000). Novel Actions of the Antitumor Drugs Vinflunine and Vinorelbine on Microtubules. *Cancer Research*, 60(18), 5045–5051.
274. Nguyen, P. H., Sigdel, K. P., Schaefer, K. G., Mensah, G. A. K., King, G. M., & Roberts, A. G. (2020). The effects of anthracycline drugs on the conformational distribution of mouse P-glycoprotein explains their transport rate differences. *Biochemical Pharmacology*, 174, 113813. <https://doi.org/10.1016/j.bcp.2020.113813>
275. Noack, A., Noack, S., Buettner, M., Naim, H. Y., & Löscher, W. (2016). Intercellular transfer of P-glycoprotein in human blood-brain barrier endothelial cells is increased by histone deacetylase inhibitors. *Scientific Reports*, 6(1), Article 1. <https://doi.org/10.1038/srep29253>
276. Nobili, S., Landini, I., Mazzei, T., & Mini, E. (2012). Overcoming tumor multidrug resistance using drugs able to evade P-glycoprotein or to exploit its expression. *Medicinal Research Reviews*, 32(6), 1220–1262. <https://doi.org/10.1002/med.20239>

277. Nosol, K., Romane, K., Irobalieva, R. N., Alam, A., Kowal, J., Fujita, N., & Locher, K. P. (2020). Cryo-EM structures reveal distinct mechanisms of inhibition of the human multidrug transporter ABCB1. *Proceedings of the National Academy of Sciences*, 117(42), 26245–26253. <https://doi.org/10.1073/pnas.2010264117>
278. O'Mara, M. L., & Mark, A. E. (2014). Structural characterization of two metastable ATP-bound states of P-glycoprotein. *PLOS ONE*, 9(3), e91916. <https://doi.org/10.1371/journal.pone.0091916>
279. Orsi, C. H., & Tanksley, S. D. (2009). Natural Variation in an ABC Transporter Gene Associated with Seed Size Evolution in Tomato Species. *PLoS Genetics*, 5(1), e1000347. <https://doi.org/10.1371/journal.pgen.1000347>
280. Pajeva, I. K., Hanl, M., & Wiese, M. (2013). Protein Contacts and Ligand Binding in the Inward-Facing Model of Human P-Glycoprotein. *ChemMedChem*, 8(5), 748–762. <https://doi.org/10.1002/cmdc.201200491>
281. Pan, L., & Aller, S. G. (2018). Allosteric Role of Substrate Occupancy Toward the Alignment of P-glycoprotein Nucleotide Binding Domains. *Scientific Reports*, 8(1), 1–14. <https://doi.org/10.1038/s41598-018-32815-2>
282. Parvez, M. M., Basit, A., Jariwala, P. B., Gáborik, Z., Kis, E., Heyward, S., Redinbo, M. R., & Prasad, B. (2021). Quantitative investigation of irinotecan metabolism, transport and gut microbiome activation. *Drug Metabolism and Disposition*. <https://doi.org/10.1124/dmd.121.000476>
283. Pasquier, J., Magal, P., Boulangé-Lecomte, C., Webb, G., & Le Foll, F. (2011a). Consequences of cell-to-cell P-glycoprotein transfer on acquired multidrug resistance in breast

cancer: A cell population dynamics model. *Biology Direct*, 6, 5. <https://doi.org/10.1186/1745-6150-6-5>

284. Pasquier, J., Magal, P., Boulangé-Lecomte, C., Webb, G., & Le Foll, F. (2011b). Consequences of cell-to-cell P-glycoprotein transfer on acquired multidrug resistance in breast cancer: A cell population dynamics model. *Biology Direct*, 6, 5. <https://doi.org/10.1186/1745-6150-6-5>

285. Patra, M., Mukhopadhyay, C., & Chakrabarti, A. (2015). Probing Conformational Stability and Dynamics of Erythroid and Nonerythroid Spectrin: Effects of Urea and Guanidine Hydrochloride. *PLOS ONE*, 10(1), e0116991. <https://doi.org/10.1371/journal.pone.0116991>

286. Patzlaff, J. S., Heide, T. van der, & Poolman, B. (2003). The ATP/Substrate Stoichiometry of the ATP-binding Cassette (ABC) Transporter OpuA *. *Journal of Biological Chemistry*, 278(32), 29546–29551. <https://doi.org/10.1074/jbc.M304796200>

287. Peeters, K., Wilmer, M. J., Schoeber, J. P., Reijnders, D., van den Heuvel, L. P., Masereeuw, R., & Levchenko, E. (2011). Role of P-Glycoprotein Expression and Function in Cystinotic Renal Proximal Tubular Cells. *Pharmaceutics*, 3(4), 782–792. <https://doi.org/10.3390/pharmaceutics3040782>

288. Pendse, S. S., Briscoe, D. M., & Frank, M. H. (2003). P-glycoprotein and alloimmune T-cell activation. *Clinical and Applied Immunology Reviews*, 4(1), 3–14. [https://doi.org/10.1016/S1529-1049\(03\)00007-2](https://doi.org/10.1016/S1529-1049(03)00007-2)

289. Pierré, A., Pérez, V., Léonce, S., Boutin, J. A., Saint-Dizier, D., Hautefaye, P., Lavielle, G., & Atassi, G. (1992). Relationship between the cellular accumulation and the cytotoxicity of S12363, a new Vinca alkaloid derivative. *Cancer Chemotherapy and Pharmacology*, 29(5), 367–374. <https://doi.org/10.1007/BF00686005>

290. Pilotto Heming, C., Muriithi, W., Wanjiku Macharia, L., Niemeyer Filho, P., Moura-Neto, V., & Aran, V. (2022). P-glycoprotein and cancer: What do we currently know? *Heliyon*, 8(10), e11171. <https://doi.org/10.1016/j.heliyon.2022.e11171>
291. Pinho, A. C., & Piedade, A. P. (2013). Zeta Potential, Contact Angles, and AFM Imaging of Protein Conformation Adsorbed on Hybrid Nanocomposite Surfaces. *ACS Applied Materials & Interfaces*, 5(16), 8187–8194. <https://doi.org/10.1021/am402302r>
292. Piotto, M., Saudek, V., & Sklenář, V. (1992). Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *Journal of Biomolecular NMR*, 2, 661–665.
293. Pokharel, D., Roseblade, A., Oenarto, V., Lu, J. F., & Bebawy, M. (2017, September 18). *Proteins regulating the intercellular transfer and function of P-glycoprotein in multidrug-resistant cancer*. <https://doi.org/10.3332/ecancer.2017.768>
294. Pomorski, T., Holthuis, J. C. M., Herrmann, A., & van Meer, G. (2004). Tracking down lipid flippases and their biological functions. *Journal of Cell Science*, 117(6), 805–813. <https://doi.org/10.1242/jcs.01055>
295. Prajoko, Y. W., & Aryandono, T. (2019). The Effect of P-Glycoprotein (P-gp), Nuclear Factor-Kappa B (Nf- κ b), and Aldehyde Dehydrogenase-1 (ALDH-1) Expression on Metastases, Recurrence and Survival in Advanced Breast Cancer Patients. *Asian Pacific Journal of Cancer Prevention : APJCP*, 20(5), 1511–1518. <https://doi.org/10.31557/APJCP.2019.20.5.1511>
296. Radeva, G., Perabo, J., & Sharom, F. J. (2005). P-Glycoprotein is localized in intermediate-density membrane microdomains distinct from classical lipid rafts and caveolar domains. *FEBS Journal*, 272, 4924–4937. <https://doi.org/10.1111/j.1742-4658.2005.04905.x>
297. Rajasekharan, A., & Gummadi, S. N. (2011). Flip-Flop of Phospholipids in Proteoliposomes Reconstituted from Detergent Extract of Chloroplast Membranes: Kinetics and

Phospholipid Specificity. *PLOS ONE*, 6(12), e28401.

<https://doi.org/10.1371/journal.pone.0028401>

298. Ramesh, R., Kozhaya, L., McKevitt, K., Djuretic, I. M., Carlson, T. J., Quintero, M. A., McCauley, J. L., Abreu, M. T., Unutmaz, D., & Sundrud, M. S. (2014). Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *The Journal of Experimental Medicine*, 211(1), 89–104. <https://doi.org/10.1084/jem.20130301>

299. Rautio, J., Meanwell, N. A., Di, L., & Hageman, M. J. (2018). The expanding role of prodrugs in contemporary drug design and development. *Nature Reviews Drug Discovery*, 17(8), Article 8. <https://doi.org/10.1038/nrd.2018.46>

300. Reddy, C. M. B., & Reddy, G. V. S. (2012). *Validated method for Vinblastin by Spectrophotometry in Bulk Drug and Pharmaceutical formulations*. 5.

301. Renard, K., & Byrne, B. (2021). Insights into the Role of Membrane Lipids in the Structure, Function and Regulation of Integral Membrane Proteins. *International Journal of Molecular Sciences*, 22(16), 9026. <https://doi.org/10.3390/ijms22169026>

302. Research, C. for D. E. and. (2021). Drug Development and Drug Interactions | Table of Substrates, Inhibitors and Inducers. *FDA*. <https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers>

303. Riou, M., Guégnard, F., Le Vern, Y., Grasseau, I., Koch, C., Blesbois, E., & Kerboeuf, D. (n.d.). Effects of cholesterol content on activity of P-glycoproteins and membrane physical state, and consequences for anthelmintic resistance in the nematode *Haemonchus contortus*. *Parasite*, 27, 3. <https://doi.org/10.1051/parasite/2019079>

304. Ritchie, T. K., Grinkova, Y. V., Bayburt, T. H., Denisov, I. G., Zolnerciks, J. K., Atkins, W. M., & Sligar, S. G. (2009). Reconstitution of membrane proteins in phospholipid bilayer

nanodiscs. In *Methods in enzymology* (19903557; 2009/11/12 ed., Vol. 464, pp. 211–231).
[https://doi.org/10.1016/S0076-6879\(09\)64011-8](https://doi.org/10.1016/S0076-6879(09)64011-8)

305. Ritchie, T. K., Kwon, H., & Atkins, W. M. (2011). Conformational analysis of human ATP-binding cassette transporter ABCB1 in lipid Nanodiscs and inhibition by the antibodies MRK16 and UIC2. *The Journal of Biological Chemistry*, 286(45), 39489–39496.
<https://doi.org/10.1074/jbc.M111.284554>

306. Roberts, A. G., Sjogren, S. E., Fomina, N., Vu, K. T., Almutairi, A., & Halpert, J. R. (2011). NMR-derived models of amidopyrine and its metabolites in complexes with rabbit cytochrome P450 2B4 reveal a structural mechanism of sequential N-dealkylation. *Biochemistry*, 50, 2123–2134. <https://doi.org/10.1021/bi101797v>

307. Robinson, K., & Tiriveedhi, V. (2020). Perplexing Role of P-Glycoprotein in Tumor Microenvironment. *Frontiers in Oncology*, 10. <https://doi.org/10.3389/fonc.2020.00265>

308. Romsicki, Y., & Sharom, F. J. (1999). The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. *Biochemistry*, 38, 6887–6896.

309. Roy, S., Kumaravel, S., Sharma, A., Duran, C. L., Bayless, K. J., & Chakraborty, S. (2020). Hypoxic tumor microenvironment: Implications for cancer therapy. *Experimental Biology and Medicine*, 245(13), 1073–1086. <https://doi.org/10.1177/1535370220934038>

310. Russell, P. L., & Sharom, F. J. (2006). Conformational and functional characterization of trapped complexes of the P-glycoprotein multidrug transporter. *Biochemical Journal*, 399, 315–323. <https://doi.org/10.1042/BJ20060015>

311. Safa, A. R. (2004). Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. *Curr Med Chem Anticancer Agents*, 4(1), 1–17.

312. Sai, Y., Nies, A. T., & Arias, I. M. (1999). Bile acid secretion and direct targeting of mdr1-green fluorescent protein from Golgi to the canalicular membrane in polarized WIF-B cells. *Journal of Cell Science*, 112 (Pt 24), 4535–4545. <https://doi.org/10.1242/jcs.112.24.4535>
313. Saib, S., & Delavenne, X. (2021). Inflammation Induces Changes in the Functional Expression of P-gp, BCRP, and MRP2: An Overview of Different Models and Consequences for Drug Disposition. *Pharmaceutics*, 13(10), Article 10. <https://doi.org/10.3390/pharmaceutics13101544>
314. Sajid, A., Lusvarghi, S., Chufan, E. E., & Ambudkar, S. V. (2018). Evidence for the critical role of transmembrane helices 1 and 7 in substrate transport by human P-glycoprotein (ABCB1). *PLOS ONE*, 13(9), e0204693. <https://doi.org/10.1371/journal.pone.0204693>
315. Salphati, L., Plise, E. G., & Li, G. (2009). Expression and activity of the efflux transporters ABCB1, ABCC2 and ABCG2 in the human colorectal carcinoma cell line LS513. *European Journal of Pharmaceutical Sciences*, 37(3), 463–468. <https://doi.org/10.1016/j.ejps.2009.04.001>
316. Sanyal, S., & Menon, A. K. (2009). Flipping lipids: Why an' what's the reason for? *ACS Chemical Biology*, 4(11), 895–909. <https://doi.org/10.1021/cb900163d>
317. Sauna, Z. E., & Ambudkar, S. V. (2007). About a switch: How P-glycoprotein (ABCB1) harnesses the energy of ATP binding and hydrolysis to do mechanical work. *Mol Cancer Ther*, 6(1), 13–23. <https://doi.org/10.1158/1535-7163.MCT-06-0155>
318. Sauna, Z. E., Müller, M., Peng, X.-H., & Ambudkar, S. V. (2002). Importance of the Conserved Walker B Glutamate Residues, 556 and 1201, for the Completion of the Catalytic Cycle of ATP Hydrolysis by Human P-glycoprotein (ABCB1). *Biochemistry*, 41(47), 13989–14000. <https://doi.org/10.1021/bi026626e>

319. Schaefer, K. G., Roberts, A. G., & King, G. M. (2023). Advantages and potential limitations of applying AFM kymograph analysis to pharmaceutically relevant membrane proteins in lipid bilayers. *Scientific Reports*, 13(1), Article 1. <https://doi.org/10.1038/s41598-023-37910-7>
320. Schinkel, A. H., & Jonker, J. W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: An overview. *Advanced Drug Delivery Reviews*, 55, 3–29.
321. Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P., & et al. (1994). Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell*, 77(4), 491–502. [https://doi.org/10.1016/0092-8674\(94\)90212-7](https://doi.org/10.1016/0092-8674(94)90212-7)
322. Schinkel, A. H., Wagenaar, E., van Deemter, L., Mol, C. A., & Borst, P. (1995). Absence of the *mdr1a* P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest*, 96(4), 1698–1705. <https://doi.org/10.1172/JCI118214>
323. Schmitt, L., & Tampé, R. (2002). Structure and mechanism of ABC transporters. *Current Opinion in Structural Biology*, 12(6), 754–760. [https://doi.org/10.1016/S0959-440X\(02\)00399-8](https://doi.org/10.1016/S0959-440X(02)00399-8)
324. Schwabedissen, H. E. M. zu, Jedlitschky, G., Gratz, M., Haenisch, S., Linnemann, K., Fusch, C., Cascorbi, I., & Kroemer, H. K. (2005). Variable Expression of Mrp2 (*abcc2*) in Human Placenta: Influence of Gestational Age and Cellular Differentiation. *Drug Metabolism and Disposition*, 33(7), 896–904. <https://doi.org/10.1124/dmd.104.003335>
325. Scian, M., Acchione, M., Li, M., & Atkins, W. M. (2014). Reaction Dynamics of ATP Hydrolysis Catalyzed by P-Glycoprotein. *Biochemistry*, 53(6), 991–1000. <https://doi.org/10.1021/bi401280v>

326. Seelig, A. (1998). A general pattern for substrate recognition by P-glycoprotein. *European Journal of Biochemistry*, 251, 252–261. <https://doi.org/10.1046/j.1432-1327.1998.2510252.x>
327. Seelig, A. (2020). P-Glycoprotein: One Mechanism, Many Tasks and the Consequences for Pharmacotherapy of Cancers. *Frontiers in Oncology*, 10, 1989. <https://doi.org/10.3389/fonc.2020.576559>
328. Seelig, A., & Landwojtowicz, E. (2000). Structure-activity relationship of P-glycoprotein substrates and modifiers. *European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences*, 12(1), 31–40.
329. Segel, I. H. (1993). *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. Wiley-Interscience.
330. Selvi, B., Patel, S., & Savva, M. (2008). Physicochemical Characterization and Membrane Binding Properties of Camptothecin. *Journal of Pharmaceutical Sciences*, 97(10), 4379–4390. <https://doi.org/10.1002/jps.21314>
331. Senapati, S., Mahanta, A. K., Kumar, S., & Maiti, P. (2018). Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduction and Targeted Therapy*, 3(1), Article 1. <https://doi.org/10.1038/s41392-017-0004-3>
332. Sever, R., & Glass, C. K. (2013). Signaling by Nuclear Receptors. *Cold Spring Harbor Perspectives in Biology*, 5(3), a016709. <https://doi.org/10.1101/cshperspect.a016709>
333. Shalinsky, D. R., Jekunen, A. P., Alcaraz, J. E., Christen, R. D., Kim, S., Khatibi, S., & Howell, S. B. (1993). Regulation of initial vinblastine influx by P-glycoprotein. *British Journal of Cancer*, 67(1), 30–36. <https://doi.org/10.1038/bjc.1993.6>

334. Shapiro, A. B., & Ling, V. (1997). Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *European Journal of Biochemistry*, 250(1), 130–137. <https://doi.org/10.1111/j.1432-1033.1997.00130.x>
335. Sharom, F. J. (1997). The P-glycoprotein efflux pump: How does it transport drugs? *The Journal of Membrane Biology*, 160(3), 161–175.
336. Sharom, F. J. (2006). Shedding light on drug transport: Structure and function of the P-glycoprotein multidrug transporter (ABCB1). *Biochemistry and Cell Biology*, 84, 979–992. <https://doi.org/10.1139/o06-199>
337. Sharom, F. J. (2007). Multidrug resistance protein: P-glycoprotein. *Drug Transporters: Molecular Characterization and Role in Drug Disposition*, 223–262.
338. Sharom, F. J. (2008). *ABC multidrug transporters: Structure, function and role in chemoresistance*.
339. Sharom, F. J. (2011a). Flipping and flopping—Lipids on the move. *IUBMB Life*, 63, 736–746. <https://doi.org/10.1002/iub.515>
340. Sharom, F. J. (2011b). The P-glycoprotein multidrug transporter. *Essays in Biochemistry*, 50, 161–178. <https://doi.org/10.1042/bse0500161>
341. Sharom, F. J. (2014). Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: Its role in modulating protein function. *Frontiers in Oncology*, 4(41), 1–19. <https://doi.org/10.3389/fonc.2014.00041>
342. Sharom, F. J., Liu, R., Romsicki, Y., & Lu, P. (1999). Insights into the structure and substrate interactions of the P-glycoprotein multidrug transporter from spectroscopic studies. *Biochim Biophys Acta*, 1461(2), 327–345.

343. Sharom, F. J., Russell, P. L., Qu, Q., & Lu, P. (2003). Fluorescence techniques for studying membrane transport proteins: The P-glycoprotein multidrug transporter. *Methods Mol Biol*, 227, 109–128. <https://doi.org/10.1385/1-59259-387-9:109>
344. Sharom, F. J., Yu, X., Chu, J. W., & Doige, C. A. (1995). Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. *Biochem J*, 308 (Pt 2), 381–390. <https://doi.org/10.1042/bj3080381>
345. Sharpe, L. J., Howe, V., Scott, N. A., Luu, W., Phan, L., Berk, J. M., Hochstrasser, M., & Brown, A. J. (2019). Cholesterol increases protein levels of the E3 ligase MARCH6 and thereby stimulates protein degradation. *The Journal of Biological Chemistry*, 294(7), 2436–2448. <https://doi.org/10.1074/jbc.RA118.005069>
346. Siarheyeva, A., Liu, R., & Sharom, F. J. (2010). Characterization of an asymmetric occluded state of P-glycoprotein with two bound nucleotides: Implications for catalysis. *Journal of Biological Chemistry*, 285, 7575–7586. <https://doi.org/10.1074/jbc.M109.047290>
347. Sigdel, K. P., Wilt, L. A., Marsh, B. P., Roberts, A. G., & King, G. M. (2018). The conformation and dynamics of P-glycoprotein in a lipid bilayer investigated by atomic force microscopy. *Biochemical Pharmacology*, 156, 302–311. <https://doi.org/10.1016/j.bcp.2018.08.017>
348. Simon, S., & Schubert, R. (2012). Inhibitory effect of phospholipids on P-glycoprotein: Cellular studies in Caco-2, MDCKII mdr1 and MDCKII wildtype cells and P-gp ATPase activity measurements. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1821(9), 1211–1223. <https://doi.org/10.1016/j.bbalip.2012.06.001>
349. Slosky, L. M., Thompson, B. J., Sanchez-Covarrubias, L., Zhang, Y., Laracuenta, M.-L., Vanderah, T. W., Ronaldson, P. T., & Davis, T. P. (2013). Acetaminophen Modulates P-Glycoprotein Functional Expression at the Blood-Brain Barrier by a Constitutive Androstane

Receptor-Dependent Mechanism. *Molecular Pharmacology*, 84(5), 774–786.
<https://doi.org/10.1124/mol.113.086298>

350. Song, J., & Melera, P. W. (2001). Transmembrane Domain (TM) 9 Represents a Novel Site in P-Glycoprotein That Affects Drug Resistance and Cooperates with TM6 to Mediate [125I]Iodoarylazidoprazosin Labeling. *Molecular Pharmacology*, 60(2), 254–261.
<https://doi.org/10.1124/mol.60.2.254>

351. Song, Y., Kenworthy, A. K., & Sanders, C. R. (2014). Cholesterol as a co-solvent and a ligand for membrane proteins. *Protein Science : A Publication of the Protein Society*, 23(1), 1–22.
<https://doi.org/10.1002/pro.2385>

352. Sonveaux, N., Vigano, C., Shapiro, A. B., Ling, V., & Ruyschaert, J. M. (1999). Ligand-mediated tertiary structure changes of reconstituted P-glycoprotein. A tryptophan fluorescence quenching analysis. *J Biol Chem*, 274(25), 17649–17654. <https://doi.org/10.1074/jbc.274.25.17649>

353. Speidel, J. T., Xu, M., & Abdel-Rahman, S. Z. (2018). Bisphenol A (BPA) and bisphenol S (BPS) alter the promoter activity of the ABCB1 gene encoding P-glycoprotein in the human placenta in a haplotype-dependent manner. *Toxicology and Applied Pharmacology*, 359, 47–54.
<https://doi.org/10.1016/j.taap.2018.09.022>

354. Srikant, S., Gaudet, R., & Murray, A. W. (2020). Selecting for Altered Substrate Specificity Reveals the Evolutionary Flexibility of ATP-Binding Cassette Transporters. *Current Biology*, 30(9), 1689–1702.e6. <https://doi.org/10.1016/j.cub.2020.02.077>

355. Srivalli, K. M. R., & Lakshmi, P. K. (2012). Overview of P-glycoprotein inhibitors: A rational outlook. *Brazilian Journal of Pharmaceutical Sciences*, 48(3), 353–367.
<https://doi.org/10.1590/S1984-82502012000300002>

356. Stewart, K. D., Johnston, J. A., Matza, L. S., Curtis, S. E., Havel, H. A., Sweetana, S. A., & Gelhorn, H. L. (2016). Preference for pharmaceutical formulation and treatment process attributes. *Patient Preference and Adherence*, 10, 1385–1399. <https://doi.org/10.2147/PPA.S101821>
357. St-Pierre, M. V., Serrano, M. A., Macias, R. I. R., Dubs, U., Hoechli, M., Lauper, U., Meier, P. J., & Marin, J. J. G. (2000). Expression of members of the multidrug resistance protein family in human term placenta. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 279(4), R1495–R1503. <https://doi.org/10.1152/ajpregu.2000.279.4.R1495>
358. Strambini, G. B., & Gonnelli, M. (2010). Fluorescence Quenching of Buried Trp Residues by Acrylamide Does Not Require Penetration of the Protein Fold. *The Journal of Physical Chemistry B*, 114(2), 1089–1093. <https://doi.org/10.1021/jp909567q>
359. Su, L., Cheng, C. Y., & Mruk, D. D. (2009). Drug transporter, P-glycoprotein (MDR1), is an integrated component of the mammalian blood–testis barrier. *The International Journal of Biochemistry & Cell Biology*, 41(12), 2578–2587. <https://doi.org/10.1016/j.biocel.2009.08.015>
360. Su, L., Jenardhanan, P., Mruk, D. D., Mathur, P. P., Cheng, Y., Mok, K.-W., Bonanomi, M., Silvestrini, B., & Cheng, C. Y. (2013). Role of P-Glycoprotein at the Blood-Testis Barrier on Adjudin Distribution in the Testis. In C. Y. Cheng (Ed.), *Biology and Regulation of Blood-Tissue Barriers* (pp. 318–333). Springer. https://doi.org/10.1007/978-1-4614-4711-5_16
361. Su, L., Mruk, D. D., Lui, W.-Y., Lee, W. M., & Cheng, C. Y. (2011). P-glycoprotein regulates blood–testis barrier dynamics via its effects on the occludin/zonula occludens 1 (ZO-1) protein complex mediated by focal adhesion kinase (FAK). *Proceedings of the National Academy of Sciences*, 108(49), 19623–19628. <https://doi.org/10.1073/pnas.1111414108>

362. Subramanian, N., Condic-Jurkic, K., & O'Mara, M. L. (2016). Structural and dynamic perspectives on the promiscuous transport activity of P-glycoprotein. *Neurochemistry International*, 98, 146–152. <https://doi.org/10.1016/j.neuint.2016.05.005>
363. Subramanian, N., Schumann-Gillett, A., Mark, A. E., & O'Mara, M. L. (2016). Understanding the accumulation of P-glycoprotein substrates within cells: The effect of cholesterol on membrane partitioning. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1858(4), 776–782. <https://doi.org/10.1016/j.bbamem.2015.12.025>
364. Summers, M. A., Moore, J. L., & McAuley, J. W. (2004). Use of Verapamil as a Potential P-Glycoprotein Inhibitor in a Patient with Refractory Epilepsy. *Annals of Pharmacotherapy*, 38(10), 1631–1634. <https://doi.org/10.1345/aph.1E068>
365. Sun, X., Tang, S., Hou, B., Duan, Z., Liu, Z., Li, Y., He, S., Wang, Q., & Chang, Q. (2021). Overexpression of P-glycoprotein, MRP2, and CYP3A4 impairs intestinal absorption of octreotide in rats with portal hypertension. *BMC Gastroenterology*, 21(1), 2. <https://doi.org/10.1186/s12876-020-01532-4>
366. Suzuki, H., & Sugiyama, Y. (2000). Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *European Journal of Pharmaceutical Sciences*, 12, 3–12.
367. Swartz, D. J., Singh, A., Sok, N., Thomas, J. N., Weber, J., & Urbatsch, I. L. (2020). Replacing the eleven native tryptophans by directed evolution produces an active P-glycoprotein with site-specific, non-conservative substitutions. *Scientific Reports*, 10(1), 3224. <https://doi.org/10.1038/s41598-020-59802-w>

368. Swartz, D. J., Weber, J., & Urbatsch, I. L. (2013). P-glycoprotein is fully active after multiple tryptophan substitutions. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1828, 1159–1168.
369. Synold, T. W., Dussault, I., & Forman, B. M. (2001). The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nature Medicine*, 7, 584–590.
370. Szaflarski, W., Sujka-Kordowska, P., Januchowski, R., Wojtowicz, K., Andrzejewska, M., Nowicki, M., & Zabel, M. (2013). Nuclear localization of P-glycoprotein is responsible for protection of the nucleus from doxorubicin in the resistant LoVo cell line. *Biomedicine & Pharmacotherapy*, 67(6), 497–502. <https://doi.org/10.1016/j.biopha.2013.03.011>
371. Szewczyk, P., Tao, H., McGrath, A. P., Villaluz, M., Rees, S. D., Lee, S. C., Doshi, R., Urbatsch, I. L., Zhang, Q., & Chang, G. (2015). Snapshots of ligand entry, malleable binding and induced helical movement in P-glycoprotein. *Acta Crystallographica Section D: Biological Crystallography*, 71(3), 732–741. <https://doi.org/10.1107/S1399004715000978>
372. Szlasa, W., Zendran, I., Zalesińska, A., Tarek, M., & Kulbacka, J. (2020). Lipid composition of the cancer cell membrane. *Journal of Bioenergetics and Biomembranes*, 52(5), 321–342. <https://doi.org/10.1007/s10863-020-09846-4>
373. Tachikawa, M., Uchida, Y., Ohtsuki, S., & Terasaki, T. (2014). Recent progress in blood–brain barrier and blood–csf barrier transport research: Pharmaceutical relevance for drug delivery to the brain. In *Drug Delivery to the Brain* (pp. 23–62). Springer.
374. Takano, M., Yumoto, R., & Murakami, T. (2006). Expression and function of efflux drug transporters in the intestine. *Pharmacology & Therapeutics*, 109, 137–161.

375. Tamai, I., & Safa, A. R. (1990). Competitive interaction of cyclosporins with the Vinca alkaloid-binding site of P-glycoprotein in multidrug-resistant cells. *The Journal of Biological Chemistry*, 265(27), 16509–16513.
376. Tanner, S. M., Staley, E. M., & Lorenz, R. G. (2013). Altered generation of induced regulatory T cells in the FVB.mdr1a^{-/-} mouse model of colitis. *Mucosal Immunology*, 6(2), 309–323. <https://doi.org/10.1038/mi.2012.73>
377. Tesauro, C., Simonsen, A. K., Andersen, M. B., Petersen, K. W., Kristoffersen, E. L., Algreen, L., Hansen, N. Y., Andersen, A. B., Jakobsen, A. K., Stougaard, M., Gromov, P., Knudsen, B. R., & Gromova, I. (2019). Topoisomerase I activity and sensitivity to camptothecin in breast cancer-derived cells: A comparative study. *BMC Cancer*, 19(1), 1158. <https://doi.org/10.1186/s12885-019-6371-0>
378. Thangapandian, S., Kapoor, K., & Tajkhorshid, E. (2020). Probing cholesterol binding and translocation in P-glycoprotein. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1862(1), 183090. <https://doi.org/10.1016/j.bbamem.2019.183090>
379. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., & Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 84(21), 7735–7738.
380. Thornton, S. J., Wong, E., Lee, S. D., & Wasan, K. M. (2008). Effect of dietary fat on hepatic liver X receptor expression in P-glycoprotein deficient mice: Implications for cholesterol metabolism. *Lipids in Health and Disease*, 7(1), 21. <https://doi.org/10.1186/1476-511X-7-21>
381. Tian, X., Li, J., Zamek-Gliszczynski, M. J., Bridges, A. S., Zhang, P., Patel, N. J., Raub, T. J., Pollack, G. M., & Brouwer, K. L. R. (2007). Roles of P-Glycoprotein, Bcrp, and Mrp2 in

Biliary Excretion of Spiramycin in Mice. *Antimicrobial Agents and Chemotherapy*, 51(9), 3230–3234. <https://doi.org/10.1128/AAC.00082-07>

382. Timsit, Y. E., & Negishi, M. (2007). CAR and PXR: The Xenobiotic-Sensing Receptors. *Steroids*, 72(3), 231–246. <https://doi.org/10.1016/j.steroids.2006.12.006>

383. Tran, N. N. B., Bui, A. T. A., Jaramillo-Martinez, V., Weber, J., Zhang, Q., & Urbatsch, I. L. (2023). Lipid environment determines the drug-stimulated ATPase activity of P-glycoprotein. *Frontiers in Molecular Biosciences*, 10. <https://www.frontiersin.org/articles/10.3389/fmolb.2023.1141081>

384. Troost, J., Lindenmaier, H., Haefeli, W. E., & Weiss, J. (2004). Modulation of Cellular Cholesterol Alters P-Glycoprotein Activity in Multidrug-Resistant Cells. *Molecular Pharmacology*, 66(5), 1332–1339. <https://doi.org/10.1124/mol.104.002329>

385. Tsujimura, S., Saito, K., Nawata, M., Nakayamada, S., & Tanaka, Y. (2008). Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis. *Annals of the Rheumatic Diseases*, 67(3), 380–388. <https://doi.org/10.1136/ard.2007.070821>

386. *Types of Chemotherapy Drugs | SEER Training*. (n.d.). Retrieved October 6, 2023, from <https://training.seer.cancer.gov/treatment/chemotherapy/types.html>

387. Ueda, K., Cardarelli, C., Gottesman, M. M., & Pastan, I. (1987). Expression of a full-length cDNA for the human “MDR1” gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proceedings of the National Academy of Sciences of the United States of America*, 84(9), 3004–3008.

388. Ulbrich, K., Holá, K., Šubr, V., Bakandritsos, A., Tuček, J., & Zbořil, R. (2016). Targeted Drug Delivery with Polymers and Magnetic Nanoparticles: Covalent and Noncovalent Approaches,

Release Control, and Clinical Studies. *Chemical Reviews*, 116(9), 5338–5431.
<https://doi.org/10.1021/acs.chemrev.5b00589>

389. Urbatsch, I. L., Beaudet, L., Carrier, I., & Gros, P. (1998). Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites. *Biochemistry*, 37(13), 4592–4602. <https://doi.org/10.1021/bi9728001>

390. Urbatsch, I. L., Sankaran, B., Weber, J., & Senior, A. E. (1995). P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J Biol Chem*, 270(33), 19383–19390.

391. Ushigome, F., Takanaga, H., Matsuo, H., Yanai, S., Tsukimori, K., Nakano, H., Uchiumi, T., Nakamura, T., Kuwano, M., Ohtani, H., & Sawada, Y. (2000). Human placental transport of vinblastine, vincristine, digoxin and progesterone: Contribution of P-glycoprotein. *European Journal of Pharmacology*, 408(1), 1–10.

392. Vahabi, S., Nazemi Salman, B., & Javanmard, A. (2013). Atomic Force Microscopy Application in Biological Research: A Review Study. *Iranian Journal of Medical Sciences*, 38(2), 76–83.

393. Vasiliou, V., Vasiliou, K., & Nebert, D. W. (2009). Human ATP-binding cassette (ABC) transporter family. *Human Genomics*, 3(3), 281. <https://doi.org/10.1186/1479-7364-3-3-281>

394. Venkitakrishnan, R. P., Benard, O., Max, M., Markley, J. L., & Assadi-Porter, F. M. (2012). Use of NMR saturation transfer difference spectroscopy to study ligand binding to membrane proteins. *Membrane Protein Structure and Dynamics: Methods and Protocols*, 47–63.

395. Verhalen, B., Ernst, S., Börsch, M., & Wilkens, S. (2012). Dynamic Ligand-induced Conformational Rearrangements in P-glycoprotein as Probed by Fluorescence Resonance Energy

Transfer Spectroscopy. *Journal of Biological Chemistry*, 287(2), 1112–1127.
<https://doi.org/10.1074/jbc.M111.301192>

396. Viegas, A., Manso, J., Nobrega, F. L., & Cabrita, E. J. (2011). Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding. *Journal of Chemical Education*, 88(7), 990–994.
<https://doi.org/10.1021/ed101169t>

397. Waghray, D., & Zhang, Q. (2017). Inhibit or evade multidrug resistance P-glycoprotein in cancer treatment. *Journal of Medicinal Chemistry*, 61(12), 5108–5121.
<https://doi.org/10.1021/acs.jmedchem.7b01457>

398. Wakabayashi, Y., Dutt, P., Lippincott-Schwartz, J., & Arias, I. M. (2005). Rab11a and myosin Vb are required for bile canaliculi formation in WIF-B9 cells. *Proceedings of the National Academy of Sciences*, 102(42), 15087–15092. <https://doi.org/10.1073/pnas.0503702102>

399. Wakabayashi, Y., Kipp, H., & Arias, I. M. (2006). Transporters on Demand: Intracellular Reservoirs and Cycling of Bile Canaliculi ABC Transporters *. *Journal of Biological Chemistry*, 281(38), 27669–27673. <https://doi.org/10.1074/jbc.R600013200>

400. Walpole, S., Monaco, S., Nepravishta, R., & Angulo, J. (2019). Chapter Twelve—STD NMR as a Technique for Ligand Screening and Structural Studies. In A. J. Wand (Ed.), *Methods in Enzymology* (Vol. 615, pp. 423–451). Academic Press. <https://doi.org/10.1016/bs.mie.2018.08.018>

401. Walton, K. D., Freddo, A. M., Wang, S., & Gumucio, D. L. (2016). Generation of intestinal surface: An absorbing tale. *Development (Cambridge, England)*, 143(13), 2261–2272.
<https://doi.org/10.1242/dev.135400>

402. Wang, J., Seebacher, N., Shi, H., Kan, Q., & Duan, Z. (2017). Novel strategies to prevent the development of multidrug resistance (MDR) in cancer. *Oncotarget*, 8(48), 84559–84571. <https://doi.org/10.18632/oncotarget.19187>
403. Ward, A. B., Szewczyk, P., Grimard, V., Lee, C.-W., Martinez, L., Doshi, R., Caya, A., Villaluz, M., Pardon, E., & Cregger, C. (2013). Structures of P-glycoprotein reveal its conformational flexibility and an epitope on the nucleotide-binding domain. *Proceedings of the National Academy of Sciences*, 110, 13386–13391. <https://doi.org/10.1073/pnas.1309275110>
404. Ward, A., Reyes, C. L., Yu, J., Roth, C. B., & Chang, G. (2007). Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proceedings of the National Academy of Sciences*, 104(48), 19005–19010. <https://doi.org/10.1073/pnas.0709388104>
405. Warrington, J. S., Greenblatt, D. J., & Moltke, L. L. von. (2004). The Effect of Age on P-Glycoprotein Expression and Function in the Fischer-344 Rat. *Journal of Pharmacology and Experimental Therapeutics*, 309(2), 730–736. <https://doi.org/10.1124/jpet.103.061234>
406. Weinstein, J. N., Collisson, E. A., Mills, G. B., Shaw, K. M., Ozenberger, B. A., Ellrott, K., Shmulevich, I., Sander, C., & Stuart, J. M. (2013). The Cancer Genome Atlas Pan-Cancer Analysis Project. *Nature Genetics*, 45(10), 1113–1120. <https://doi.org/10.1038/ng.2764>
407. Wen, P.-C., Mahinthichaichan, P., Trebesch, N., Jiang, T., Zhao, Z., Shinn, E., Wang, Y., Shekhar, M., Kapoor, K., Chan, C. K., & Tajkhorshid, E. (2018). Microscopic view of lipids and their diverse biological functions. *Current Opinion in Structural Biology*, 51, 177–186. <https://doi.org/10.1016/j.sbi.2018.07.003>
408. White, S. H., & Wimley, W. C. (1999). MEMBRANE PROTEIN FOLDING AND STABILITY: Physical Principles. *Annual Review of Biophysics and Biomolecular Structure*, 28(1), 319–365. <https://doi.org/10.1146/annurev.biophys.28.1.319>

409. Wilkens, S. (2015). Structure and mechanism of ABC transporters. *F1000Prime Reports*, 7. <https://doi.org/10.12703/P7-14>
410. Wilt, L. A., Nguyen, D., & Roberts, A. G. (2017). Insights into the molecular mechanism of triptan transport by P-glycoprotein. *Journal of Pharmaceutical Sciences*, 106, 1670–1679. <https://doi.org/10.1016/j.xphs.2017.02.032>
411. Wouters, O. J., McKee, M., & Luyten, J. (2020). Estimated Research and Development Investment Needed to Bring a New Medicine to Market, 2009-2018. *JAMA*, 323(9), 844–853. <https://doi.org/10.1001/jama.2020.1166>
412. Wu, C.-P., Calcagno, A. M., & Ambudkar, S. V. (2008). Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: Evaluation of current strategies. *Current Molecular Pharmacology*, 1(2), 93–105. <https://doi.org/10.2174/1874-470210801020093>
413. Wu, J., Lin, N., Li, F., Zhang, G., He, S., Zhu, Y., Ou, R., Li, N., Liu, S., Feng, L., Liu, L., Liu, Z., & Lu, L. (2016). Induction of P-glycoprotein expression and activity by Aconitum alkaloids: Implication for clinical drug–drug interactions. *Scientific Reports*, 6. <https://doi.org/10.1038/srep25343>
414. Xia, D., Zhou, F., & Esser, L. (2019). Emerging consensus on the mechanism of polyspecific substrate recognition by the multidrug transporter P-glycoprotein. *Cancer Drug Resistance*, 2(3), 471–489. <https://doi.org/10.20517/cdr.2019.22>
415. Xu, Y. D., Lai, R. Y., Procházková, E., & Stenzel, M. H. (2021). Saturation Transfer Difference NMR Spectroscopy for the Elucidation of Supramolecular Albumin–Polymer Interactions. *ACS Macro Letters*, 10(7), 819–824. <https://doi.org/10.1021/acsmacrolett.1c00270>

416. Yammine, A., Gao, J., & Kwan, A. H. (2019). Tryptophan Fluorescence Quenching Assays for Measuring Protein-ligand Binding Affinities: Principles and a Practical Guide. *Bio-Protocol*, 9(11), e3253. <https://doi.org/10.21769/BioProtoc.3253>
417. Yan, A., Jia, Z., Qiao, C., Wang, M., & Ding, X. (2020). Cholesterol metabolism in drug-resistant cancer (Review). *International Journal of Oncology*, 57(5), 1103–1115. <https://doi.org/10.3892/ijo.2020.5124>
418. Yang, S.-T., Kreutzberger, A. J. B., Lee, J., Kiessling, V., & Tamm, L. K. (2016). The Role of Cholesterol in Membrane Fusion. *Chemistry and Physics of Lipids*, 199, 136–143. <https://doi.org/10.1016/j.chemphyslip.2016.05.003>
419. Yano, K., Tomono, T., & Ogiwara, T. (2018). Advances in Studies of P-Glycoprotein and Its Expression Regulators. *Biological and Pharmaceutical Bulletin*, 41(1), 11–19. <https://doi.org/10.1248/bpb.b17-00725>
420. Yau, J. C., Yap, Y. Y., Buzdar, A. U., Hortobagyi, G. N., Bodey, G. P., & Blumenschein, G. R. (1985). A comparative randomized trial of vinca alkaloids in patients with metastatic breast carcinoma. *Cancer*, 55(2), 337–340. [https://doi.org/10.1002/1097-0142\(19850115\)55:2<337::aid-cnrcr2820550205>3.0.co;2-u](https://doi.org/10.1002/1097-0142(19850115)55:2<337::aid-cnrcr2820550205>3.0.co;2-u)
421. Ye, J., & Berg, B. (2004). Crystal structure of the bacterial nucleoside transporter TssX. *The EMBO Journal*, 23(16), 3187–3195. <https://doi.org/10.1038/sj.emboj.7600330>
422. Yihong Qiu, Geoff G.Z. Zhang, Yisheng Chen, Lawrence Yu, & Rao V. Mantri. (2016). *Developing Solid Oral Dosage Forms—2nd Edition*. Academic Press. <https://www.elsevier.com/books/developing-solid-oral-dosage-forms/qiu/978-0-12-802447-8>

423. Yin, J., & Wang, J. (2016). Renal drug transporters and their significance in drug–drug interactions. *Acta Pharmaceutica Sinica. B*, 6(5), 363–373. <https://doi.org/10.1016/j.apsb.2016.07.013>
424. Young, S. D., Whissell, M., Noble, J. C. S., Cano, P. O., Lopez, P. G., & Germond, C. J. (2006). Phase II Clinical Trial Results Involving Treatment with Low-Dose Daily Oral Cyclophosphamide, Weekly Vinblastine, and Rofecoxib in Patients with Advanced Solid Tumors. *Clinical Cancer Research*, 12(10), 3092–3098. <https://doi.org/10.1158/1078-0432.CCR-05-2255>
425. Yu, J., Ge, J., Heuveling, J., Schneider, E., & Yang, M. (2015). Structural basis for substrate specificity of an amino acid ABC transporter. *Proceedings of the National Academy of Sciences of the United States of America*, 112(16), 5243–5248. <https://doi.org/10.1073/pnas.1415037112>
426. Zabolina, O. A., Zhang, N., & Weerts, R. (2021). Polysaccharide Biosynthesis: Glycosyltransferases and Their Complexes. *Frontiers in Plant Science*, 12, 625307. <https://doi.org/10.3389/fpls.2021.625307>
427. Zacchia, M., Capolongo, G., Rinaldi, L., & Capasso, G. (2018). The importance of the thick ascending limb of Henle’s loop in renal physiology and pathophysiology. *International Journal of Nephrology and Renovascular Disease*, 11, 81–92. <https://doi.org/10.2147/IJNRD.S154000>
428. Zappe, K., & Cichna-Markl, M. (2020). Aberrant DNA Methylation of ABC Transporters in Cancer. *Cells*, 9(10), 2281. <https://doi.org/10.3390/cells9102281>
429. Zawadzka, I., Jeleń, A., Pietrzak, J., Żebrowska-Nawrocka, M., Michalska, K., Szmajda-Krygier, D., Mirowski, M., Łochowski, M., Kozak, J., & Balcerczak, E. (2020). The impact of ABCB1 gene polymorphism and its expression on non-small-cell lung cancer development,

progression and therapy – preliminary report. *Scientific Reports*, 10, 6188.
<https://doi.org/10.1038/s41598-020-63265-4>

430. Zhang, J., Sun, T., Liang, L., Wu, T., & Wang, Q. (2013). Drug promiscuity of P-glycoprotein and its mechanism of interaction with paclitaxel and doxorubicin. *Soft Matter*, 10(3), 438–445. <https://doi.org/10.1039/C3SM52499J>

431. Zhang, Q., He, J., Zhang, M., & Ni, P. (2015). A polyphosphoester-conjugated camptothecin prodrug with disulfide linkage for potent reduction-triggered drug delivery. *Journal of Materials Chemistry B*, 3(24), 4922–4932. <https://doi.org/10.1039/C5TB00623F>

432. Zhang, Y., & Benet, L. Z. (2001). The gut as a barrier to drug absorption. *Clinical Pharmacokinetics*, 40, 159–168.

433. Zhao, Y., Wan, B., Zhang, T., Xu, Y., Liu, H., Lv, T., Zhang, F., Zhan, P., & Song, Y. (2019). Irinotecan, topotecan, paclitaxel or docetaxel for second-line treatment of small cell lung cancer: A single-center retrospective study of efficiency comparison and prognosis analysis. *Translational Lung Cancer Research*, 8(6). <https://doi.org/10.21037/tlcr.2019.10.21>

434. Zhao, Z., Ukidve, A., Kim, J., & Mitragotri, S. (2020). Targeting Strategies for Tissue-Specific Drug Delivery. *Cell*, 181(1), 151–167. <https://doi.org/10.1016/j.cell.2020.02.001>

435. Zhou, S.-F. (2008). Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*, 38, 802–832.
<https://doi.org/10.1080/00498250701867889>

436. Zlokovic, B. V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron*, 57, 178–201.

437. Zoghbi, M. E., Mok, L., Swartz, D. J., Singh, A., Fendley, G. A., Urbatsch, I. L., & Altenberg, G. A. (2017). Substrate-induced conformational changes in the nucleotide-binding

domains of lipid bilayer-associated P-glycoprotein during ATP hydrolysis. *J Biol Chem*.

<https://doi.org/10.1074/jbc.M117.814186>

ABBREVIATIONS

ABC: ATP-binding cassette

Amp: amplitude

AMP: adenosine monophosphate

AMPPNP: adenosine 5'-(β,γ -imido)triphosphate

ATP: adenosine triphosphate

BBB: blood-brain barrier

BTB: blood-testis barrier

CHAPS: 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate

CNS: central nervous system

CNS: Crystallography & NMR System

CS: chemical shift

DDM: *n*-dodecyl- β -*D*-maltoside

DEAE: diethylaminoethyl cellulose

E_{vdw} : Van der Waals energies

HSQC: heteronuclear single quantum correlation

ICLs: intracellular loops

IHs: intracellular helices

K_D : Dissociation constant

K_{Pi} : potassium phosphate

K_{SV} : Stern-Volmer quenching constant

NATA: N-acetyl-L-tryptophanamide

NBDs: nucleotide-binding domain

Ni-NTA: nickel-nitrilotriacetic acid

NMR: nuclear magnetic resonance

NOE: intramolecular nuclear Overhauser effect

Pgp: P-glycoprotein

Pi: inorganic phosphate

PRE: paramagnetic relaxation enhancement

SAR: structure-activity relationship

SOI: sphere of influence

STD: saturation transfer difference

STDD: saturation transfer double difference

SXR: steroid and xenobiotic receptor

TM: transmembrane helix

TMD: transmembrane domain

VO₃: orthovanadate

WATERGATE: water suppression by gradient tailored excitation.