Characterizing drug interactions in the substrate binding pocket of the
P-glycoprotein multidrug efflux pump

by

David Ward

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Biophysics

Guelph, Ontario, Canada
© David Ward, February, 2012
ABSTRACT

CHARACTERIZING DRUG INTERACTIONS IN THE SUBSTRATE BINDING POCKET OF THE P-GLYCOPROTEIN MULTIDRUG EFFLUX PUMP

David Ward
University of Guelph, 2012

Advisor:
Professor F.J. Sharom

P-glycoprotein (Pgp, ABCB1) is a polyspecific efflux transporter implicated in multidrug resistance in human cancers. In this study, tetramethylrhodamine-5-carbonyl azide (AzTMR) was covalently crosslinked to the Pgp drug binding pocket with a stoichiometry of 1. The Pgp-AzMTR adduct was functionally equivalent to unlabelled Pgp and retained its ability to transport Hoechst 33342. The binding site of AzTMR in Pgp was nonpolar, with a similar environment to that of propanol. Pgp-AzMTR could bind a second drug molecule, with a higher affinity for H-site drugs and lower affinity for other R-site drugs. Unlabelled Pgp interacted with dimeric versions of known Pgp modulators, binding them with higher affinity than the monomer. These compounds were also found to either stimulate or inhibit Pgp ATPase activity depending on the concentration. Pgp-AzMTR was able to bind dimeric drugs, indicating that 3 substrate moieties can fit into the binding pocket.
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Frances Sharom, for the opportunity to complete the research contained in this thesis and very helpful suggestions throughout the drafting phase and defence preparation. I would also like to thank the members of my advisory committee, Dr. Rod Merrill and Dr. Leonid Brown, for their support throughout my research phase and for contributing to the editing of my thesis and Dr. George Hauraz for reading my thesis and serving on my examination committee. I would like to extend my gratitude to our collaborators, Dr. Christine Hrycyna and Dana Emmert at Purdue University, who provided samples used in this research and were always eager to discuss our experimental results. I am grateful to the many past and present members of our laboratory, who provided much technical guidance and friendship.

Last but by no means least is the recognition of the continuous support and encouragement throughout my undergraduate and graduate studies given by my parents, grandparents, family and many friends. This thesis would not have been possible without them and I am extremely grateful for their contributions to my life.
### TABLE OF CONTENTS

**ABSTRACT**

**ACKNOWLEDGMENTS** ........................................................................................................................................ iii

**TABLE OF CONTENTS** ........................................................................................................................................ iv

**LIST OF TABLES** ............................................................................................................................................... viii

**LIST OF FIGURES** ........................................................................................................................................... ix

**GLOSSARY OF ABBREVIATIONS** ................................................................................................................ xii

1 **INTRODUCTION** ......................................................................................................................................... 1

1.1 P-glycoprotein ............................................................................................................................................. 2

1.1.1 ABC superfamily of proteins .................................................................................................................. 2

1.1.2 Physiological role of P-glycoprotein ....................................................................................................... 3

1.1.3 P-glycoprotein and multidrug resistance ................................................................................................. 4

1.2 Structure of Pgp ........................................................................................................................................... 6

1.2.1 Topology of Pgp ..................................................................................................................................... 6

1.2.2 Conserved regions of Pgp ..................................................................................................................... 6

1.2.3 X-ray crystal structure of Pgp ............................................................................................................... 8

1.3 Interaction of drugs and nucleotides with Pgp ......................................................................................... 12

1.3.1 Pgp substrate specificity: substrates and modulators ............................................................................ 12

1.3.2 Nature of the Pgp drug binding pocket .................................................................................................. 15

1.3.3 Binding and hydrolysis of nucleotides .................................................................................................. 17

1.4 Transport of drugs by Pgp .......................................................................................................................... 20

1.4.1 Models of drug transport: hydrophobic vacuum cleaner and flippase .................................................. 20

1.4.2 Catalytic cycle of Pgp ............................................................................................................................ 22

1.4.3 Pgp drug transport sites ....................................................................................................................... 25
1.5 Rationale and research objectives ................................................................. 27

2 MATERIALS AND METHODS ........................................................................... 30

2.1 Materials ........................................................................................................ 31

2.2 Pgp purification.................................................................................................. 31
  2.2.1 Plasma membrane (PM) isolation from CH^{R}B30 cells ....................... 32
  2.2.2 Detergent extraction of Pgp ................................................................. 33
  2.2.3 Pgp purification ..................................................................................... 34
  2.2.4 Protein assays ........................................................................................ 34

2.3 Determination of Pgp ATPase activity ........................................................... 35

2.4 Preparation and characterization of Pgp-AzTMR ............................................ 37
  2.4.1 Crosslinking of AzTMR to Pgp ............................................................ 37
  2.4.2 Optimization of crosslinking conditions ............................................... 38
  2.4.3 Stoichiometry of AzTMR linkage ........................................................ 38
  2.4.4 Fluorescence properties of Pgp-AzTMR drug adduct ......................... 39
  2.4.5 Polarity of the AzTMR binding site in Pgp .......................................... 39

2.5 Determination of Pgp drug binding affinity .................................................... 40
  2.5.1 Pgp intrinsic Trp fluorescence quenching ............................................. 40
  2.5.2 Enhanced H33342 and H33258 fluorescence on Pgp binding ............. 42

2.6 Pgp transport assay ........................................................................................ 43
  2.6.1 Functional reconstitution of Pgp ........................................................... 43
  2.6.2 Real-time fluorescence transport assay ................................................. 44

3 CROSSLINKING OF AZTMR TO PURIFIED PGP .............................................. 45

3.1 Introduction ..................................................................................................... 47

3.2 Covalent crosslinking of AzTMR to Pgp ....................................................... 49

3.3 Optimization of crosslinking conditions ....................................................... 50
### 3.4 Stoichiometry of covalent linkage of AzTMR to Pgp .......................... 57

### 3.5 Discussion ..................................................................................................... 60

### 4 BIOCHEMICAL CHARACTERIZATION OF PGP-AZTMR ................. 63

#### 4.1 Introduction ................................................................................................. 65

#### 4.2 Determining the polarity of the local environment of bound AzTMR in Pgp .......................................................... 68

#### 4.3 Interaction of R-site drugs with Pgp and Pgp-AzTMR ......................... 70

- **4.3.1** Effect on Pgp ATPase activity .............................................................. 70
- **4.3.2** Binding affinity of Pgp for R-site drugs .............................................. 72

#### 4.4 Interaction of H-site drugs with Pgp and Pgp-AzTMR ......................... 76

- **4.4.1** Effects on Pgp ATPase activity ............................................................ 76
- **4.4.2** Binding affinity of Pgp for H-site drugs .............................................. 76

#### 4.5 Binding of clinically important drugs to Pgp and Pgp-AzTMR ........... 80

#### 4.6 Pgp-AzTMR drug transport ....................................................................... 81

#### 4.7 Discussion ..................................................................................................... 83

### 5 BINDING OF DRUG MONOMERS AND DIMERS TO PGP/PGP-AZTMR AND EFFECTS ON ATPASE ACTIVITY .......................... 86

#### 5.1 Introduction ................................................................................................. 88

#### 5.2 Abacavir monomer and dimers ................................................................. 91

- **5.2.1** Binding affinity of Pgp and Pgp-AzTMR for abacavirs ...................... 91

#### 5.3 Quinine monomer and dimers ................................................................. 95

- **5.3.1** Binding affinity of Pgp and Pgp-AzTMR for quinines ...................... 95
- **5.3.2** Effect on ATPase activity ................................................................. 95

#### 5.4 Quetiapine monomer and dimers ............................................................ 99

- **5.4.1** Binding affinity of Pgp and Pgp-AzTMR for quetiapines .............. 99
- **5.4.2** Effect on ATPase activity ................................................................. 103
5.5 Discussion ................................................................................................... 107

6 SUMMARY AND CONCLUSIONS ....................................................................... 110

6.1 Summary and conclusions ........................................................................... 112

6.2 Suggestions for future work ........................................................................ 115

REFERENCES ...................................................................................................... 117
LIST OF TABLES

Table 1.1  Representative Pgp substrates ................................................................. 13
Table 1.2  Representative modulators of Pgp activity .............................................. 13
Table 4.1  Binding affinity of Pgp and Pgp-AzTMR for R-site drugs ................................. 74
Table 4.2  Binding affinity of Pgp and Pgp-AzTMR for Hoechst compounds .................. 78
Table 4.3  Binding affinity of Pgp and Pgp-AzTMR for clinically important drugs  .......... 80
Table 5.1  Binding affinity of Pgp and Pgp-AzTMR for abacavirs ............................... 93
Table 5.2  Binding affinity of Pgp and Pgp-AzTMR for quinines ............................... 97
Table 5.3 IC50 values for inhibition of Pgp and Pgp-AzTMR ATPase activity by quetiapines 101
Table 5.4  Binding affinity of Pgp and Pgp-AzTMR for quetiapines ............................... 101

viii
LIST OF FIGURES

Figure 1.1  Domain organization of ABC proteins ................................................... 2
Figure 1.2  Topology of Pgp showing 11 Trp residues ............................................. 7
Figure 1.3  X-ray crystal structures of full length ABC transporters ..................... 10
Figure 1.4  Coordination of ATP in the NBDs of ABC transporters ....................... 18
Figure 1.5  The role of Pgp as a hydrophobic vacuum cleaner and a flippase ................................................................. 21
Figure 1.6  Summary of the Pgp transport cycle..................................................... 26
Figure 2.1  SDS-PAGE of Pgp purified from CHR8B30 cells .................................. 35
Figure 3.1  Fluorescence properties of Pgp and Pgp-AzTMR ................................ 51
Figure 3.2  Crosslinking timecourse of AzTMR to Pgp ......................................... 53
Figure 3.3  Effect of different initial AzTMR concentrations on crosslinking ................. 55
Figure 3.4  Effect of ATP and DTE on Pgp-AzTMR crosslinking ............................ 56
Figure 3.5  Stoichiometry of AzTMR labelling of Pgp ........................................... 58
Figure 4.1  Some Pgp substrates used in experiments with Pgp and Pgp-AzTMR ..................... 67
Figure 4.2  Lippert plot of AzTMR in solvents of varying polarity .............................. 69
Figure 4.3  Effect of AzTMR on ATPase activity and binding of Pgp and Pgp-AzTMR ................................................................. 71
Figure 4.4  Effect of TMR and TMR-E on ATPase activity of Pgp and Pgp-AzTMR ................................................................. 73
Figure 4.5  TMR and TMR-E binding to Pgp and Pgp-AzTMR .................................. 75
Figure 4.6  Effect of H33342 on ATPase activity of Pgp and Pgp-AzTMR .................... 77
Figure 4.7  Binding affinity of Pgp for H33342 determined by Trp quenching and Hoechst fluorescence enhancement ........................... 79
Figure 4.8  H33342 transport by Pgp and Pgp-AzTMR proteoliposomes
........................................................................................................ 82

Figure 5.1  Structures of monomers and dimers used in this study .......... 89

Figure 5.2  Quenching of Pgp and Pgp-AzTMR Trp fluorescence
by abacavirs .......................................................................................... 92

Figure 5.3  Drug dimer binding to Pgp-AzTMR ........................................ 94

Figure 5.4  Quenching of Pgp and Pgp-AzTMR Trp fluorescence
by quinines ............................................................................................ 96

Figure 5.5  Effect of quinines on ATPase activity of Pgp and Pgp-
AzTMR ................................................................................................. 98

Figure 5.6  Binding of quetiapine monomer and dimers to Pgp ............... 100

Figure 5.7  Binding of quetiapine monomer and dimers to Pgp-
AzTMR ............................................................................................... 102

Figure 5.8  ATPase activity profiles of quetiapine C2 and C7
dimers with Pgp and Pgp-AzTMR ......................................................... 105

Figure 5.9  ATPase activity of quetiapine and IC₅₀ of quetiapine
dimers ................................................................................................. 106
## GLOSSARY OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>5'-adenylimido-diphosphate</td>
</tr>
<tr>
<td>ATP-γ-S</td>
<td>adenosine-5'-O-(3-thio-triphosphate)</td>
</tr>
<tr>
<td>AzTMR</td>
<td>tetramethylrhodamine-5-carboxyl azide</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein (ABCG2)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMPC</td>
<td>dimyristoylphosphatidylcholine</td>
</tr>
<tr>
<td>DTE</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>extracellular loop</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>H33258</td>
<td>Hoechst 33258</td>
</tr>
<tr>
<td>H33342</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>H-site</td>
<td>Hoechst 33342 transport site</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinesulfonic acid</td>
</tr>
<tr>
<td>ICL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>IAAP</td>
<td>iodoarylazidoprazosin</td>
</tr>
<tr>
<td>LDS-751</td>
<td>2-[4-(4-[dimethylamino]phenyl)-1,3-butadienyl]-3-ethylbenzothiazolium perchlorate</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>α-MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MRP1</td>
<td>multidrug resistance-associated protein 1 (ABCC1)</td>
</tr>
<tr>
<td>MTS</td>
<td>methanethiosulfonate</td>
</tr>
<tr>
<td>NATA</td>
<td>N-acetyl-L-tryptophanamide</td>
</tr>
<tr>
<td>NB</td>
<td>nucleotide-binding</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide-binding domain</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Pgp-AzTMR</td>
<td>Pgp-AzTMR covalent adduct</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Pgp-mock</td>
<td>mock crosslinked Pgp</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationship</td>
</tr>
<tr>
<td>R101</td>
<td>rhodamine 101</td>
</tr>
<tr>
<td>R110</td>
<td>rhodamine 110</td>
</tr>
<tr>
<td>R123</td>
<td>rhodamine 123</td>
</tr>
<tr>
<td>R6G</td>
<td>rhodamine 6G</td>
</tr>
<tr>
<td>R-site</td>
<td>rhodamine 123 transport site</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TMEA</td>
<td>tris-(2-maleimidoethyl)amine</td>
</tr>
<tr>
<td>TMR</td>
<td>tetramethylrosamine</td>
</tr>
<tr>
<td>TMR-E</td>
<td>tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TMR-M</td>
<td>tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>$V_i$</td>
<td>ortho-vanadate</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION
1.1 P-GLYCOPROTEIN

1.1.1 ABC superfamily of proteins

The mammalian multidrug efflux pump P-glycoprotein (Pgp) is a membrane transporter that is the most widely studied member of the ABC superfamily of proteins. This 170 kDa product of the *MDR1/ABCB1* gene (*abcb1a* and *abcb1b* in mice) was originally identified in Chinese hamster ovary cells selected for colchicine resistance, which developed a reduced rate of drug uptake (1). The ABC superfamily is the largest protein family in many organisms, and its members carry out a wide variety of transport processes in both prokaryotes and eukaryotes (2). In fact, over 80 ABC proteins exist in *E. coli* (3) and 49 have been identified in the human genome (4). ABC proteins are typically composed of four core domains: two α-helical transmembrane domains (TMDs) which together form the substrate translocation pathway, and two cytoplasmic nucleotide-binding domains (NBDs) that hydrolyze ATP to power drug transport (Figure 1.1) (5). In eukaryotes, these four domains are commonly expressed as a single polypeptide, presumed to have arisen from an internal duplication, while in prokaryotes

![Figure 1.1 Domain organization of ABC proteins](image-url)
they can be synthesized as two or four separate subunits.

Bacterial ABC proteins can be importers or exporters, while mammalian ABC proteins are exclusively exporters; all are active transporters, moving their substrates up a concentration gradient (6). Some ABC transporters have broad substrate specificity, such as the mammalian multidrug efflux pumps, Pgp, multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP). Others, however, specialize in the transport of a specific substrate; for example, maltose permease, which imports maltose in bacteria. Several ABC proteins have been studied extensively because of their role in human health and disease; mutations in 17 different human ABC transporters have been implicated in specific disease processes (7). However, the physiological role of many human ABC proteins remains unknown.

1.1.2  Physiological role of P-glycoprotein

Low levels of Pgp expression have been observed in most tissues, however, it is most abundant in the apical membranes of polarized epithelial cells with excretory functions, such as those in the intestine, kidney, liver and pancreas (8;9). Pgp is also located in the endothelial cell membranes of blood-tissue barriers, including the blood-brain barrier (BBB), placenta, blood-testis, blood-ovary and blood-nerve barriers (10). The exact physiological role of Pgp in each of these tissues is not known with certainty, and is complicated by the multiplicity of apparent substrates, however, it may play a role in protecting the body from toxic compounds, both endogenous and xenobiotics. Pgp can prevent substrates from being absorbed in the intestine, and mediates their excretion via the kidney/liver/pancreatic ducts. In the barrier tissues, Pgp is oriented such that it would expel substrates into the blood, which also supports the idea that Pgp prevents cytotoxic
compounds from entering sensitive organs (11). Double-knockout mice (abcb1a/b\(^{-/-}\)) that are null for Pgp display a normal phenotype and are fertile, however, when exposed to the anti-parasitic agent ivermectin, they die from its toxicity, while wild-type mice do not (12). The knockout mice could not expel the neurotoxic ivermectin which accumulated in their brains, whereas wild-type mice were protected by the presence of Pgp in the BBB. Moreover, collie dogs with a naturally occurring frameshift mutation that results in lack of Pgp expression display high sensitivity to ivermectin relative to wild-type dogs (13). The tissues where Pgp is found are important barriers in the absorption, distribution and elimination of drugs, and it is therefore not surprising that Pgp affects the bioavailability and pharmacokinetics of many clinically important drugs, including those used in chemotherapy treatments for a variety of human diseases and cancers.

1.1.3 P-glycoprotein and multidrug resistance

Tumour cells can become simultaneously resistant to a wide range of anti-cancer drugs in a condition known as multidrug resistance (MDR). Thirteen ABC transporters, including Pgp, have been reported to be involved in its development (14). MDR can arise from a variety of cellular mechanisms including altered drug metabolism, p53 mutation and altered DNA repair processes, but drug efflux resulting from Pgp overexpression is thought to be one of the main causes (15;16). In fact, Pgp expression levels are correlated with resistance to several anti-cancer drugs in the U.S. National Cancer Institute’s collection of tumour cell lines, and it is believed to contribute to chemotherapy drug resistance in at least 50% of human cancers (17;18). Some cancers, including those of the colon, liver, pancreas and kidney, tend to be inherently drug-resistant, while others such as leukemias, myelomas, ovarian and breast cancers most often develop MDR subsequent
to chemotherapy treatment. Pgp has been reported in many of these tumours and in some cases, its expression increases after one or more rounds of chemotherapy (19).

Following retroviral transfer of the human MDR1 gene into the bone marrow cells of mice, the cells became resistant to the cytotoxic drug taxol, demonstrating the ability of Pgp to confer drug resistance in vivo (20). Since Pgp is the most prevalent cause of MDR, it soon emerged as an attractive target of drugs aimed at reversing MDR in human cancers. This led to the development of Pgp modulators, compounds that are capable of reversing drug resistance. Modulators used in combination with chemotherapy drugs would improve the uptake and distribution of those compounds into tumour cells, making them more cytotoxic. The early use of modulators in clinical cancer treatment was somewhat disappointing as poor clinical trial design, patient selection and toxicity led to sub-optimal outcomes for patients (21). Modulators have shown some positive effects in a few cases, including the treatment of acute myeloid leukemia with the Pgp modulator cyclosporin A in combination with cytarabine and daunorubicin (22), and more selective, less cytotoxic agents have been developed (23;24). Tumours expressing elevated levels of Pgp prior to chemotherapy tend to be less responsive to Pgp inhibitors than tumours that develop MDR over the course of treatment (25), highlighting the need for more research into the role of Pgp in MDR and the design of modulators to combat it. However, many recent clinical trials with new “third generation” modulators have failed, leading to doubt about the ability of this strategy to improve the outcome of chemotherapy treatment (26).
1.2 Structure of Pgp

1.2.1 Topology of Pgp

Pgp is a “full transporter”, a single 1280 amino acid polypeptide organized into two homologous halves thought to have arisen from gene duplication. The two halves are connected by a cytoplasmic 60-amino acid flexible linker region which can be phosphorylated, and both the N- and C-termini face the cytoplasm. The topology of Pgp (Figure 1.2) was confirmed biochemically by analyzing the immunofluorescence of inserted epitope tags and extensive biotinylation of cysteines inserted at specific locations in Cys-less Pgp (27;28). Each half of the protein was found to contain 6 membrane embedded α-helices connected by intracellular loops (ICL) and extracellular loops (ECL), which form the substrate translocation pathway, followed by a cytosolic NBD. Pgp is N-glycosylated on its first ECL, and glycosylation plays a role in trafficking of Pgp to the cell surface (29). However, it is not necessary for transport, since unglycosylated Pgp mutants are able to transport substrates normally (30;31). Pgp contains 11 Trp residues (Figure 1.2) distributed throughout the protein, with 8 in the N-terminal half and 3 in the C-terminal half (32), which provide a source of intrinsic protein fluorescence. In addition, there are 7 Cys residues in Pgp, including one in each NBD, which are useful for labelling Pgp with specific Cys-reactive probes (33).

1.2.2 Conserved regions of Pgp

In light of the common transport and ATP hydrolysis functions of ABC proteins, it is not surprising that they share common features. There is a low level of sequence similarity in the TMDs, which is likely due to each one having evolved as the translocation pathway for a particular substrate(s). However, the NBDs are very well
Figure 1.2  Topology of Pgp showing 11 Trp residues

Topology diagram showing the arrangement of the TMDs and NBDs of Pgp in the plasma membrane. The 12 transmembrane (TM) helices are labelled TM1-12, and the N- and C-termini and two NBDs are located in the cytoplasm. The ICLs and ECLs are not labelled but are shown as loops connecting the TMDs and NBDs. The 11 Trp amino acid residues in Pgp are identified where they occur in the sequence.
conserved between different transporters and across species, which is expected given the common mechanism of ATP-driven transport. NBD1 and NBD2 have 25% and 28% sequence identity, and 50% similarity, respectively, across all ABC proteins, and a number of conserved motifs are present \(^{(34,35)}\). The NBDs of many proteins that bind or hydrolyze ATP or GTP contain a Walker A (GXXGXS/TS) and Walker B (hhhhD; h=hydrophobic amino acid) motif \(^{(36)}\), but the signature C motif (LSGGQQ/R/KQR) of Pgp is unique to the ABC superfamily \(^{(37)}\). Pgp also contains highly conserved amino acids in the A-loop, Q-loop, H-loop, D-loop and Pro-loop of its NBDs. These loops, along with the Walker A, Walker B and signature C motifs, are involved in the binding and coordination of ATP, its divalent cation Mg\(^{2+}\), and the water molecule involved in ATP hydrolysis \(^{(38)}\). The Q-loop and Pro-loop may also be involved in interdomain communication, coupling ATP hydrolysis to the TMDs \(^{(39)}\).

1.2.3 X-ray crystal structure of Pgp

Until recently, there was no high resolution structural information available for Pgp, and researchers had to rely on comparisons to known bacterial ABC transporter structures and low resolution electron microscopic structures. However, in 2009, the first medium-high resolution X-ray crystal structure of Pgp was published, showing the apo- and drug-bound states at 3.8-4.4 Å resolution \(^{(40)}\). The crystal structures validate many findings of the Pgp homology models and lower resolution structures, but have been criticized widely for physiological relevance.

High resolution X-ray crystallographic structures are available for the bacterial lipid A flippase MsbA in several nucleotide-bound states \(^{(41)}\), and the transporter Sav1866 from \textit{S. aureus} with bound ADP and 5'-adenylimido-diphosphate (AMP-PNP) (Figure
1.3A and B) \((42;43)\). Both MsbA and Sav1866 show the highest sequence similarity with Pgp out of all ABC transporters with known high resolution structures, and each contain 12 TM helices which enclose their central cavity. The structures of both bacterial transporters indicate that some TM helices (TM1-2 in Sav1866 and TM4-5 in MsbA) in each half of the protein associate with those in the other half, which introduces a twist into the overall structure. When nucleotides are present, the NBDs of both transporters are closely associated, and the TMD bundles form a funnel that is closed at the cytoplasmic side. However, in the absence of nucleotide, two states were observed for MsbA, in which the NBDs were separated to varying degrees, and the TMD bundle appeared to open to the cell interior \((41)\). These findings led to a proposal that the TM helices can exist in an outward- or inward-facing conformation in the presence or absence of nucleotide, respectively, and provided for a mechanism whereby the binding of ATP drives the structural reorganization necessary for substrate translocation \((44)\). Indeed, it is now believed that ATP binding induces NBD dimerization, and reorientation of the TMDs so they face the extracellular environment to release their substrates. ATP hydrolysis is proposed to return the TMDs to the inward-facing state to start the transport process over again \((45)\). Homology models of Pgp were constructed based on the structures of bacterial transporters, and predicted that Pgp could also undergo rearrangement of its TMDs in a nucleotide-dependent manner \((46;47)\). Moreover, biochemical cross-linking experiments with Pgp showed that TMD2 contacts NBD1, indicating that it likely has a domain architecture similar to that of Sav1866 \((48)\).

Several EM structures of Pgp have been reported over the years \((49-53)\), with the use of 2D crystals \((50)\) and cryo-EM leading to increased resolution \((51)\). These
The X-ray crystal structures of three ABC transporters are shown. _Salmonella typhimurium_ MsbA with AMP-PNP bound (3B60) is shown in A (41), while _S. aureus_ Sav1866 with ADP bound (2HYD) is shown in B (43); each half of the transporter is coloured differently. Three crystal structures of Pgp, with its N-terminal half in purple and C-terminal half in blue, are shown in C (3G5U), in the absence of substrate, D (3G60), with a single molecule of bound QZ59-RRR (green), and E (3G61) with two molecules of bound QZ59-SSS (yellow and orange) (40). The approximate location of the lipid bilayer is indicated by the blue band.

**Figure 1.3  X-ray crystal structures of full length ABC transporters**

The X-ray crystal structures of three ABC transporters are shown. _Salmonella typhimurium_ MsbA with AMP-PNP bound (3B60) is shown in A (41), while _S. aureus_ Sav1866 with ADP bound (2HYD) is shown in B (43); each half of the transporter is coloured differently. Three crystal structures of Pgp, with its N-terminal half in purple and C-terminal half in blue, are shown in C (3G5U), in the absence of substrate, D (3G60), with a single molecule of bound QZ59-RRR (green), and E (3G61) with two molecules of bound QZ59-SSS (yellow and orange) (40). The approximate location of the lipid bilayer is indicated by the blue band.
structures have confirmed many conclusions from the homology models, specifically, that the NBDs close in the presence of ATP and that the TM helices reorganize during this process. One of the added discoveries was that further conformational changes in the TMDs may take place after ATP hydrolysis, as shown in an EM structure of the ADP-vanadate post-hydrolysis state (50).

The recent 3.8-4.4 Å X-ray crystal structures of mouse Pgp provided a new template to validate all the prior biochemical and structural evidence (40). The protein was crystallized nucleotide-free in the apo form and in complex with two stereoisomers of a cyclic peptide inhibitor, cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS) (Figure 1.3C-E). All three forms were catalytically active. All structures were inward-facing and showed the two 6-helix bundles enclosing a large 6000 Å³ internal cavity with pseudo-two-fold symmetry. Interestingly, the two helical bundles contained TMs 1-3, 6, 10, 11 and 4, 5, 7-9, 12, confirming that “helix-swapping” takes place in the two halves, as with MsbA and Sav1866. The Pgp structures agree with much of the prior work on ABC transporter structure, along with the biochemical and biophysical data on Pgp. The crystal structure showed one molecule of QZ59-SSS bound at a “middle” site in the binding pocket (Figure 1.3D), and two molecules of QZ59-RRR bound at “upper” and “lower” sites which overlapped the middle site (Figure 1.3E). These structures confirm earlier reports that two drug molecules can bind to Pgp simultaneously (54;55). The binding pocket is made up of a core set of hydrophobic and aromatic amino acid residues from both TMDs, yet each binding site contains several unique residues specific to that site. An ~9 Å wide “portal” is found on each side of the
protein, embedded within the membrane, possibly providing access for the entry of hydrophobic substrates directly from the inner leaflet.

All three crystal structures show that the NBDs are widely separated by ~30 Å, which is perhaps not surprising given the absence of nucleotide, but raises questions as to the physiological validity of this state. Indeed, reactions towards this structure have been mixed, and criticisms have arisen directed at the absence of ATP, and the absence of any significant conformational changes on drug binding (56). Additional structures with bound nucleotide will be needed to piece together a deeper understanding of Pgp’s catalytic and transport mechanism.

1.3 Interaction of Drugs and Nucleotides with Pgp

1.3.1 Pgp substrate specificity: substrates and modulators

Pgp can transport hundreds of structurally diverse substrates across the membrane using the energy from ATP hydrolysis, and theoretical studies suggest its substrates could number into the thousands (57). A Pgp substrate is typically a large, hydrophobic, amphipathic, polyaromatic compound with a positively-charged quaternary nitrogen atom, although not all of its substrates meet these requirements (11,58,59). Most substrates were identified by their reduced cytotoxicity in cells overexpressing Pgp, rather than by direct binding or transport measurements. Substrates include Vinca alkaloids and other chemotherapeutic drugs, peptides, steroids, natural products, HIV protease inhibitors, and fluorescent dyes (11,59,60) (Table 1.1). When binding of these structurally unrelated compounds is measured, the range in Kd values spans several orders of magnitude, indicating that Pgp can discriminate between its substrates (61).
### Table 1.1 Representative Pgp substrates

<table>
<thead>
<tr>
<th>Anticancer drugs</th>
<th>Fluorescent compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vinca</em> alkaloids (vinblastine)</td>
<td>tetramethylrosamine</td>
</tr>
<tr>
<td>anthracyclines (daunorubicin)</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>rhodamine 123</td>
</tr>
<tr>
<td></td>
<td>LDS-751</td>
</tr>
<tr>
<td>HIV protease inhibitors</td>
<td>Linear/cyclic peptides</td>
</tr>
<tr>
<td>ritonavir</td>
<td>ALLN</td>
</tr>
<tr>
<td>saquinavir</td>
<td>NAc-LLY-amide</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Cardiac glycosides</td>
</tr>
<tr>
<td>actinomycin D</td>
<td>digoxin</td>
</tr>
<tr>
<td>Steroids</td>
<td>Detergents</td>
</tr>
<tr>
<td>aldosterone</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>nonylphenol ethoxylate</td>
</tr>
</tbody>
</table>

### Table 1.2 Representative modulators of Pgp activity

<table>
<thead>
<tr>
<th>Calcium channel blockers</th>
<th>Immunosuppressive agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>verapamil</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>nifedipine</td>
<td>PSC833</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Calmodulin antagonists</td>
</tr>
<tr>
<td>erythromycin</td>
<td>trifluoperazine</td>
</tr>
<tr>
<td>valinomycin</td>
<td>chlorpromazine</td>
</tr>
<tr>
<td>Steroids</td>
<td>Miscellaneous</td>
</tr>
<tr>
<td>progesterone</td>
<td>LY335979 (Zosuquidar)</td>
</tr>
<tr>
<td>tamoxifen</td>
<td>XR-9576 (Tariquidar)</td>
</tr>
<tr>
<td></td>
<td>OC144-093 (Ontogen)</td>
</tr>
<tr>
<td></td>
<td>quinidine</td>
</tr>
<tr>
<td></td>
<td>propafenone</td>
</tr>
<tr>
<td></td>
<td>dipyridamole</td>
</tr>
</tbody>
</table>
The large number and structural variability of Pgp substrates have made it difficult to explain how so many compounds can interact with a single protein. Substrates have been proposed to bind to Pgp mainly through van der Waals forces, hydrophobic interactions, H-bonding and π-π stacking interactions with Trp and Tyr residues (62). Indeed, in the Pgp X-ray crystal structure, interactions between the hexapeptide substrate and aromatic side chains from the TMDs are evident (40). Genetic mutations at specific amino acid residues have been shown to alter the binding affinity of one substrate to Pgp, but have no effect on others (63;64). These findings led to a proposed induced-fit mechanism for drug binding to Pgp: the substrate enters the large binding pocket and establishes a small core set of interactions, followed by small TM helix and side chain movements to create a sub-set of specific interactions unique to that substrate (65). This proposal is supported by the X-ray structure of Pgp, where each of the three substrate molecules are bound to different overlapping locations, and contain both common and unique binding contacts (40).

Attempts have been made to establish a pharmacophore model for Pgp, but because of the great diversity of substrates, this has proved difficult. The presence of H-bond donors (66) and aromatic Trp residues (some of which are sensitive to substrate binding (32)) in the TM regions of Pgp suggested that interactions with these residues might be common to substrate binding (62). A quantitative structure-activity relationship (QSAR) for Pgp substrates was proposed, where two or three H-bond acceptors/electron donors and certain hydrophobic groups with a fixed spatial arrangement form a basal set of common structural features (66;67).
Pgp also interacts with another class of structurally diverse compounds known as modulators. Modulators (also known as reversers or chemosensitizers), block the action of Pgp, and when co-administered with a cytotoxic Pgp substrate, can reverse MDR (68). The exact mechanism of action of modulators is not well understood, but it is thought that they either interact competitively with substrates for the Pgp binding pocket (69), interact with another site (70), or engage the protein in a futile cycle of ATP hydrolysis and transport (71). Indeed, some modulators have a higher spontaneous flip-flop rate from the outer to the inner membrane leaflet, so that they re-engage with Pgp faster than substrates with slow flip-flop rates, and are transported out again (72).

1.3.2 Nature of the Pgp drug binding pocket

The exact nature of the drug binding site(s) within Pgp is still under debate (73-75), however, it is thought to be both large and flexible. The binding pocket was localized to the cytoplasmic half of the protein TMDs by FRET studies (76;77), and biochemical and crosslinking analysis (78-80), which is consistent with the X-ray crystal structure (40). The crystal structure also confirmed that the drug binding site is funnel shaped, which had previously been proposed through crosslinking studies (55;81;82). Simultaneous binding of tris-(2-maleimidoethyl)amine (TMEA) and a second drug substrate was reported by Loo and Clarke (55), and photoaffinity labelling studies with azidopine suggested that Pgp was capable of binding drug in two different regions (83), indicating that it may possess multiple binding sites. An additional photoaffinity labelling study found seven distinct, but partially interacting, binding sites within the TMDs (84), and binding experiments showed that several compounds which interact with Pgp are able to influence each other allosterically (85). To explain these data, Globisch et al. constructed
a homology model of Pgp based on Sav1866 to predict the number and nature of Pgp drug binding sites (86). Interestingly, they proposed that Pgp contained 40 different binding sites clustered into 3 larger binding regions: one at the TMD-cytoplasm interface and the other two in the TMDs in the membrane interior. Amino acid mutations in the TMD-cytoplasm region showed that this location is important in substrate specificity (63;87-89), and the other two TMD regions are important for drug binding and allosteric communication with the NBDs (90;91).

Early reports indicated that the drug binding pocket may be accessible to the aqueous environment (50;92), however, fluorescence techniques have shown that the H- and R-sites are hydrophobic in nature (54;77). Extensive crosslinking studies carried out with Cys-less Pgp suggested that the binding pocket was formed at the interface between the two TMDs, with residues contributed mainly from TM helices 4, 5, and 6 from the N-terminal TMD, and TM helices 9, 10, 11, and 12 from the C-terminal TMD (79;93). In the X-ray crystal structure of Pgp, both peptide stereoisomers bind between TM helices 6 and 12 (40). The binding of QZ59-RRR to the middle site takes place mostly through hydrophobic interactions with residues in TMs 1, 5, 6, 7, 11, and 12. The molecule of QZ59-SSS occupying the upper site is also surrounded by hydrophobic amino acids from TMs 1, 2, 6, 7, 11, and 12, while the molecule occupying the lower site is surrounded by residues from TMs 1, 5, 6, 7, 8, 9, 11, and 12, including three polar residues (40). Many of the interactions with nearby amino acids are common to all three bound drug molecules, but each site has a small subset of unique contacts, and it is likely that even within the large membrane-embedded binding pocket, there are polarity differences in each of the local environments.
1.3.3 Binding and hydrolysis of nucleotides

The binding and hydrolysis of nucleotides by ABC transporters occurs at the catalytic and helical domains of the NBDs, which are separated by the Q- and Pro-loops (39). The catalytic domain of each NBD contains the Walker A, Walker B, and H-loop, while the signature C motif is located in the helical domain (Figure 1.4A). The catalytic events that take place in the NBDs include the binding of ATP, NBD dimerization, ATP hydrolysis, separation of the NBDs, release of P$_i$ and lastly, release of ADP. Several different models have been developed, based on many structural, biochemical and mutational studies, to explain these events and how they are coupled to the TMDs to drive the transport of substrates.

Since the overall architecture of ABC transporter NBDs is conserved among the ABC superfamily, the X-ray crystal structure of the E. coli toxin transporter, haemolysin B (HlyB) has been used as a model of ATP binding (39). The binding of ATP is mediated by the Walker A and Walker B motif of one NBD and the signature C motif of the other NBD, so that two molecules of ATP bind along the interface of the two NBDs to form a nucleotide sandwich dimer (Figure 1.4B). Residues in the signature C motif are thought to stabilize the formation of the NBD dimer in the presence of ATP by interacting with the $\gamma$-phosphate. It is generally thought that the NBDs are closely associated in the absence of ATP (52,94,95) so that only small conformational changes take place during ATP-driven dimerization, however, the NBDs of MsbA are apparently widely separated (41). The NBD subunit from MJ0796, made catalytically inactive through mutation (E171Q), was still able to bind two molecules of ATP (96), as was HlyB (97). Mutation of a conserved His residue in the H-loop of HlyB (H662A) confirmed that it is essential
Figure 1.4  Coordination of ATP in the NBDs of ABC transporters

(A) The coordination of nucleotide (ATP) by the amino acid residues in the highly conserved motifs of the NBDs is shown in this schematic representation (taken from (98) with permission). (B) The X-ray crystal structure of the NBDs of the catalytically inactive (H662A) mutant of HlyB with bound nucleotide (taken from (99) with permission).
for activity. This residue is proposed to be a “linchpin” of catalytic activity, necessary for inter-NBD communication (99). Additional mutations in the Walker A and Walker B motifs have been shown to reduce catalytic turnover while maintaining some degree of ATP binding (100;101).

ATP and ADP bind to Pgp with similar affinity (0.2-0.5 mM) to the nonhydrolysable nucleotide analog AMP-PNP (32). However, the fluorescent nucleotide analogs TNP-ADP and TNP-ATP bind with much higher affinity, most likely because of additional hydrophobic interactions (102). Nucleotides can bind to Pgp in the absence or presence of substrates, but substrate binding typically has some effect on ATPase activity. Pgp possesses high constitutive ATPase activity which can be stimulated, inhibited, or left unchanged by the addition of substrates and modulators (103;104). Some compounds stimulate Pgp ATPase activity at low concentrations, and inhibit it at higher concentrations, which led to the idea that Pgp may possess stimulatory and inhibitory drug binding sites (105). It is currently unknown why Pgp possesses metabolically inefficient high basal ATPase activity.

Senior et al. originally proposed, based on vanadate-trapping experiments, that both NBDs can bind and hydrolyze ATP, but only one molecule of ATP is hydrolyzed at a given time, in what is known as the alternating sites model (106). This model assumes that once the first ATP molecule is hydrolyzed at NBD1, and ADP and Pᵢ are released, the second ATP molecule is then hydrolyzed at NBD2. Other experimental evidence supports this model: inactivation of one NBD by mutation (107), nucleotide trapping (108) or labelling with N-ethylmaleimide (109) resulted in a protein that was catalytically inactive, yet still able to bind ATP at the other NBD. Moreover, the NBDs appeared to be
functionally equivalent, as inactivation took place equally at each NBD. More recent work showed, however, that Pgp is able to simultaneously bind two molecules of the non-hydrolysable analogue adenosine-5’-O-(3-thio-triphosphate) (ATP-γ-S) with different affinities ($K_d = 0.74$ mM and 6 $\mu$M) demonstrating asymmetric “occlusion” of ATP during the catalytic cycle (110). It is proposed that two molecules of ATP initially bind loosely at each NBD. The NBDs then dimerize into the occluded conformation, with the tightly bound ATP committed to hydrolysis (101;111). The tightly bound ATP passes through the transition state and is hydrolyzed, and transport results from either formation of the occluded state or ATP hydrolysis, via the coupling helices (111). The next molecule of ATP is hydrolyzed at the other NBD, completing the cycle of ATP hydrolysis.

1.4 TRANSPORT OF DRUGS BY Pgp

1.4.1 Models of drug transport: hydrophobic vacuum cleaner and flippase

The original model of membrane transport was that of a classic pump that moved polar substrates from the aqueous phase, through its hydrophilic interior directly to the aqueous phase on the other side of the membrane. However, since hydrophobic compounds with low water solubility were discovered to be Pgp substrates, the hydrophobic vacuum cleaner and lipid flippase models were proposed (112). The hydrophobic vacuum cleaner model suggests that Pgp binds drugs within the membrane and expels them into the extracellular medium, while in the flippase model it flips drugs from the inner to the outer leaflet of the membrane (Figure 1.5). The difficulty in distinguishing experimentally between these two models lies in the rapid partitioning of drugs between the aqueous phase and the membrane, so that drug extruded into the
Figure 1.5  The role of Pgp as a hydrophobic vacuum cleaner and a flippase

As a hydrophobic vacuum cleaner, Pgp binds drugs within the membrane and expels them into the extracellular medium, while as a flippase, it flips drugs from the inner leaflet to the outer leaflet of the membrane.
extracellular medium will immediately re-partition into the outer leaflet.

Both models are supported by the finding that substrates gain access to the Pgp binding site from the cytoplasmic leaflet of the membrane (76,77) and the X-ray crystal structure, which shows a side portal that could provide direct access to the membrane (40). The polarity of the local environment around LDS-751 bound to Pgp is also not compatible with an aqueous channel of the type found in the classic pump model (76). Furthermore, drugs with high lipid-water partition coefficients seem to have higher Pgp binding affinity (113), suggesting that the membrane may serve to concentrate substrates for presentation to Pgp, and the movement of drugs from the membrane into the binding pocket is a nearly isoenergetic process (114).

In support of the flippase model, Pgp shares 78% sequence similarity with MDR3 (ABCB4), a PC lipid flippase found in the liver that is able to transport some Pgp substrates (115). Pgp has also been shown to flip various fluorescently-labelled phospholipids (116) and simple glycosphingolipids (117) in a process that is dependent on ATP and inhibited by vanadate. Moreover, drugs appear to compete with Pgp lipid flippase activity, suggesting that lipids and drugs may follow the same transport pathway. More sophisticated time-resolved techniques will be needed to determine with absolute certainty whether Pgp is actually a lipid flippase or hydrophobic vacuum cleaner.

1.4.2 Catalytic cycle of Pgp

The transport cycle of Pgp involves coupling ATP turnover with substrate translocation and its exact nature is still the subject of much controversy. Several steps
have been studied individually, but how they work together to actively transport many
different types of compounds is not yet known. The binding and hydrolysis of ATP, the
release of ADP/P_i, and the binding of substrates have all been shown to result in
conformational changes in the TMDs of Pgp (118;119). Furthermore, covalent
crosslinking of the TMDs was found to reversibly inhibit ATPase activity, indicating that
the TMDs and NBDs are somehow coupled (120). The presence of a high affinity
stimulatory drug binding site (“on”) and a low affinity inhibitory binding site (“off”) has
been suggested to explain how drug binding can either stimulate or inhibit ATPase
activity (121). However, it is not known whether the conformational changes in the
TMDs are driven by ATP binding or ATP hydrolysis.

The X-ray crystal structures of many ABC proteins show short α-helices extending
from the TMDs into the interface between the helical and catalytic subdomains of the
NBDs. These coupling helices are thought to be the link in transmitting conformational
changes in the NBDs resulting from ATP hydrolysis to the TMDs. In Pgp, the coupling
helix from one half interacts with the opposing NBD, and could be responsible for
changing the TMDs to the outward-facing conformation upon NBD dimerization (40;45).
The conformational changes could be initiated either through the binding of ATP, or
formation/breakdown of the ATP hydrolysis transition state. Deletion mutations in these
regions resulted in Pgp that could not transport substrates and did not show drug-
stimulated increases in ATPase activity (122).

The ATP switch model, which was proposed as a mechanism for substrate
translocation by all ABC proteins, suggests that resting state Pgp has a high affinity for
substrates and low affinity for ATP, and that ATP binding, not hydrolysis, provides the
energy for drug transport (123). In this model, drug binding induces ATP binding by communicating with the NBDs, resulting in NBD dimerization and formation of the occluded nucleotide state. This in turn, causes conformational changes in the TMDs that expose the substrate to the extracellular environment. The affinity of Pgp for substrate is lower in this conformation, resulting in substrate release. Hydrolysis of ATP and dissociation of ADP/P$_i$ would then reset Pgp back to its resting inward-facing state to repeat the process again. However, Pgp substrates are not absolutely required for NBD dimerization and ATP hydrolysis (102;124) and other studies have suggested that ATP hydrolysis, rather than ATP binding, causes reorientation of the drug binding site (125). Therefore, the ATP switch model may not be an accurate description for transport mediated by Pgp.

The alternating sites model, suggested by Senior et al., proposes that the energy for drug transport is provided by relaxation of the high-energy transition state formed during ATP hydrolysis. The NBDs are suggested to bind and hydrolyze ATP in an alternating fashion, with one ATP used to transport one substrate molecule (106). The partition model, which was developed by al-Shawi and co-workers from steady-state thermodynamic analyses of ATP hydrolysis in the presence and absence of substrates, is an expanded view of the alternating sites model (124). It suggests that Pgp participates in either a basal ATPase activity cycle, which is uncoupled from drug transport, or a drug-coupled catalytic cycle that results in drug transport. In both the coupled and basal cycles, the rate-limiting step was ATP hydrolysis, and the energy released from one round of ATP hydrolysis was sufficient to rehydrate a single drug molecule. The hydrolysis of ATP was proposed to move substrates from a high affinity “on” site to a drug-unloading
“off” site, equivalent to the inward-facing and outward-facing conformations of the Pgp TMDs, respectively (124). Similar free energy analysis was used to explain drug-induced inhibition of Pgp ATPase activity, and suggested that it could result from inhibition of drug release from the lower affinity “off” site in the presence of high drug concentrations (124).

A summary of the Pgp drug translocation pathway (Figure 1.6) was published by Callaghan et al. and describes four key stages (126). The first step is loading of Pgp with drug and nucleotide; it is suggested that drug binding occurs first and enhances ATP binding, consistent with the ATP switch model, resulting in NBD dimerization. In the second step, the TMDs reorient from a high- to low-affinity binding site (or inward- to outward-facing conformation), resulting in substrate release (i.e. transport). ATP hydrolysis occurs in the third step and finally, ADP and P_i are released causing the NBDs to separate and the TMDs return to the inward-facing (high-affinity) conformation. Despite many studies on drug translocation by Pgp, the structural mechanism by which the NBDs and TMDs are coupled, and whether ATP binding or hydrolysis provides the energy for transport, are still controversial.

1.4.3 Pgp drug transport sites

The ability of Pgp to transport so many structurally dissimilar compounds has resulted in its binding site(s) being the subject of much investigation. Shapiro and Ling demonstrated the presence of two functional drug transport sites by measuring the transport kinetics of rhodamine 123 (R123) and Hoechst 33342 (H33342) in native PM vesicles (127). The R-site, which showed a preference for R123 transport, and the H-site, which had a preference for H33342, exhibited positive cooperativity for transport. R123,
The four stages of transport by Pgp as proposed by Callaghan et al. (126). Drug and nucleotide loading promote NBD dimerization (1); the TMDs reorient to the outward-facing low affinity conformation, releasing the drug substrate on the other side of the membrane (2); nucleotide hydrolysis and release (3); resetting the TMDs to the high affinity inward-facing conformation (4).

daunorubicin, and doxorubicin stimulated H33342 transport and inhibited R123 transport, while H33342, colchicine, and quercetin stimulated R123 transport and inhibited H33342 transport (127). They later went on to propose the existence of a third transport site, the P-site (specific for progesterone and prazosin) which also interacts allosterically with the R- and H-sites (128).

The bidirectional transport of R123 and H33342 was measured in polarized monolayers of MDCK cells transfected with human MDR1 by Tang et al. (129). They found that R123 stimulated H33342 efflux, but contrary to the observations of Shapiro and Ling (127), H33342 inhibited R123 efflux. Moreover, H33342 did not affect the
cellular uptake of R123, and R123 actually decreased H33342 uptake. It is not known why this study was unable to confirm positive cooperativity between the R- and H-sites.

LDS-751 was shown by Shapiro and Ling to stimulate H33342 transport, suggesting that it interacted with the R-site (130), however, when both LDS-751 and R123 were found to simultaneously bind to Pgp (54), this cast some doubt on its previous classification. It is possible that the R-site is a large binding region capable of binding both drugs. Recent work in our laboratory with Pgp reconstituted into DMPC liposomes found that H33342 could stimulate tetramethylrosamine (TMR) transport, but H33342 transport was not stimulated or inhibited by several rhodamine dyes (Balpreet Vinepal, 2008, M.Sc. thesis, University of Guelph). Furthermore, H33342 was found to inhibit TMR transport at higher concentrations, suggesting a complex interaction between the two substrates. LDS-751 was also shown to inhibit both TMR and H33342 transport, indicating it may not bind exclusively to the R-site as previously proposed.

These conflicting observations may be due to the different environment of Pgp in each case, but they do indicate that the allosteric interactions in the Pgp drug binding pocket are likely much more complex than the original R-site/H-site model had suggested.

1.5 RATIONALE AND RESEARCH OBJECTIVES

Pgp can bind a number of substrates from the Hoechst and rhodamine families of compounds that are chemically and structurally distinct. In addition, the transport of LDS-751 and the somewhat contradictory results obtained in R123 and H33342 transport competition experiments suggest that the original H- and R-site model is overly
simplistic. Therefore, a better understanding of how multiple substrates are accommodated simultaneously in the Pgp binding pocket, and how they affect each other’s binding and transport is needed. The interactions between drug combinations are especially important for chemotherapy treatment, where drug cocktails of Pgp substrates are frequently used.

The goals of this project were to better characterize the drug-drug interactions inside the substrate binding pocket of Pgp, and to better understand how the pocket accommodates multiple drugs, and how they mutually affect each other’s binding (and transport). Specifically, a drug molecule was covalently crosslinked to a defined site in the substrate-binding pocket, and the interactions with a second drug were examined. This approach avoids the problem of multiple reversible drug equilibria that was a complication in previous studies. Moreover, drug monomers and dimers were used to provide information on the size and capacity of the binding pocket. In order to accomplish this, Pgp was purified in detergent solution or reconstituted into proteoliposomes. Drug binding was determined by fluorescence spectroscopy, where both the intrinsic Trp fluorescence of Pgp, and the fluorescence of the drug molecules were measured.
Chapter 2

MATERIALS AND METHODS
2.1 **MATERIALS**

Disodium ATP, \(N\)-acetyl-L-tryptophanamide (NATA), dithioerythritol (DTE), sodium ortho-vanadate (V\(_i\)) and 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (Oakville, ON). Concanavalin A (ConA)-Sepharose 4B was obtained from GE Healthcare (Baie d’Urfé, QC). The Econo10-DG pre-packed gel filtration columns were purchased from Bio-Rad (Mississauga, ON). Protease inhibitors and bovine serum albumin (BSA) (crystallized and lyophilized) were obtained from Roche Diagnostics (Laval, QC). Liquid scintillation vials, DMSO and acrylamide were purchased from Fisher Scientific (Whitby, ON). Hoechst 33342 (H33342), Hoechst 33258 (H33258), 2-[4-(4-[dimethylamino]phenyl)-1,3-butadienyl]-3-ethylbenzothiazolium perchlorate (LDS-751), rhodamine 123 (R123), rhodamine 6G (R6G), rhodamine 101 (R101), rhodamine 110 (R110), tetramethylrosamine (TMR), tetramethylrhodamine methyl ester (TMR-M), tetramethylrhodamine ethyl ester (TMR-E), tetramethylrhodamine-5-carbonyl azide (AzTMR) were purchased from Molecular Probes (Eugene, OR). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from MP Biomedicals (Solon, OH). Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL). The abacavir (131), quinine (132) and quetiapine dimers were synthesized and provided by Christine Hrycyna (Purdue University, Richmond, IN). All solvents used were of HPLC grade.

2.2 **PGP PURIFICATION**

The Pgp used in all experiments was obtained from Chinese hamster ovary cells (CH\(_k\)B30) which were selected for resistance to colchicine (133) using a modification of
a 3-step purification procedure, as previously described (32;134;135). The amount of protein and ATPase activity were determined after each purification step, and samples were kept on ice throughout the entire process unless otherwise indicated.

2.2.1 Plasma membrane (PM) isolation from CHR30B30 cells

The CHR30B30 cells were grown in the presence of 30 μg/mL colchicine, 2 mM L-glutamine, streptomycin (1 mg/mL) and penicillin (1000 i.u./mL) in α-minimal essential medium (α-MEM) containing 10% heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, UT) at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested, resuspended in freezing medium (α-MEM/10% DMSO) and stored at -80°C until their use. Cells were stored no more than 6 months before use. For each batch of PM prepared, approximately 6 tubes of 1 × 1010 CHR30B30 cells each were thawed and each tube was washed 3 times with cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.0 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and centrifuged for 5 min at 4,000g at 4°C. The supernatant washes were discarded and the pellets resuspended in ~15 mL of homogenizing buffer (10 mM Tris, 0.25 M sucrose, 0.2 mM CaCl2, 0.02% (w/v) NaN3, pH 7.5), combined into 3 tubes and centrifuged for 5 min at 4,000g at 4°C. The pellet in each tube was resuspended in ~20 mL cold homogenizing buffer containing protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 50 μg/mL phenylmethylsulfonylfluoride (PMSF)).

The 3 tubes of cells were lysed using nitrogen cavitation by passage through a Yeda press for 2 min at 400-500 psi. After 3 passes, 2 mL of 10 mM EDTA and ~15 mL of post-homogenizing buffer (10 mM Tris, 0.025 M sucrose, 0.02% (w/v) NaN3, pH 7.5) was added to each tube of cell lysate. Each ~36 mL tube of cell homogenate was divided
evenly between two open-top polycarbonate ultracentrifuge tubes and layered on top of 10 mL of a 35% sucrose cushion buffer (10 mM Tris, 35% (w/w) sucrose, 1 mM EDTA, 0.02% (w/v) NaN₃, pH 7.5). The six tubes were ultracentrifuged in an SW-32 Ti rotor at 27,400 rpm (128,000g) for 1 h at 4°C. Following centrifugation, the PM layer that settled at the interface was collected with a glass pipette and pooled into two 70 Ti screw-cap polycarbonate centrifuge tubes. The PM was washed twice with ~20 mL of freezing buffer (10 mM Tris, 0.25 M sucrose, 0.02% (w/v) NaN₃, pH 7.5) and centrifuged for 35 min at 41,000 rpm (173,000g) at 4°C in a 70 Ti rotor. The supernatant washes were discarded and the final pellet resuspended in ~6 mL of freezing buffer using a 1 mL syringe and 26%G needle. Aliquots were removed to determine protein concentration and ATPase activity and the purified PM was stored at -80°C.

2.2.2 Detergent extraction of Pgp

Pgp was extracted from the isolated CH₃B30 PM by a 3-step detergent extraction with the zwitterionic detergent CHAPS. The frozen PM was thawed and centrifuged for 35 min at 41,000 rpm (173,000g) at 4°C in a 70 Ti rotor. The PM pellet was resuspended in 100 μL of solubilization buffer I (15 mM CHAPS, 20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.4) for every 1 mg of PM protein and incubated on ice for 30 min with periodic swirling. The CHAPS-extracted PM was centrifuged for 30 min at 41,000 rpm (173,000g) at 4°C in a 70 Ti rotor. The supernatant was collected (S1) and the remaining pellet resuspended in 1 mL of solubilization buffer II (45 mM CHAPS, 20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.4) for every 10 mg of PM protein using a 1 mL syringe and 26%G needle. The solution was transferred to microfuge tubes, mixed gently on a Nutator for 30 min and centrifuged for 15 min at 13,000 rpm at 4°C in a
microcentrifuge. The supernatants were collected and pooled (S2). The S1 and S2 fractions were assayed for protein content and ATPase activity. The S2 fraction was diluted to a final CHAPS concentration of 15 mM, aliquoted into 1 mL fractions and frozen at -80°C until purification.

2.2.3  Pgp purification

Pgp was purified from the S2 fraction by affinity chromatography. The ConA-Sepharose 4B column (1 × 20 cm) was blocked with ~35 mL of α-MEM and washed with ~100 mL equilibration buffer (2 mM CHAPS, 20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 5 mM DTE, pH 7.4); 8 mL of S2 was loaded and run through with the same buffer. The flow rate was 1 drop/5 s and approximately 20-25 0.5 mL fractions were collected. The column was then washed with ~100 mL 20 mM HEPES buffer, ~50 mL of 0.5 M glucose in 20 mM HEPES buffer, ~50 mL of 20% ethanol in storage buffer (0.1 M sodium acetate, 1 mM CaCl₂, 1 mM MgCl₂, 0.02% (w/v) NaN₃, pH 6.0) to remove bound glycoproteins, and ~100 mL of storage buffer in which it was then stored.

The column fractions were monitored for absorbance at 280 nm, protein content, and ATPase assay. Fractions with A₂₈₀>1 were pooled and assayed again for protein content and ATPase activity. The pooled Pgp was 90-95% pure, with a concentration of 0.2-0.3 mg/mL and ATPase activity of 1.5-2.5 μmol/min/mg (Figure 2.1). The purified protein was aliquoted and stored at 4°C.

2.2.4  Protein assays

The protein concentration was determined at various stages of Pgp purification and in the experiments thereafter by the methods of Bradford (136) and Peterson (137), which
Figure 2.1  SDS-PAGE of Pgp purified from CH\textsuperscript{R}B30 cells

Lane 1 contains 10 μL of Bio-Rad Kaleidoscope Precision Plus protein standards (250, 150, 100, 75, 50, 37 and 25 kDa, from top to bottom) and Lane 2 was loaded with ~10 μg of ConA-purified Pgp.

were modified for use in a 96-well plate using BSA as the protein standard. Samples were always assayed in triplicate.

2.3  Determination of Pgp ATPase Activity

The specific ATPase activity of Pgp at various stages of purification and following functional reconstitution into DMPC liposomes was determined using a colorimetric assay that measures the release of inorganic phosphate from ATP (138). An inorganic phosphate standard curve was prepared by adding 100 μL samples (0-20 nmol KH\textsubscript{2}PO\textsubscript{4}) to a 96-well plate. Protein samples containing Pgp were diluted in either 2 mM CHAPS/20 mM HEPES buffer or 20 mM HEPES buffer to a volume of 90 μL. Blank samples (buffer only) were also prepared and 10 μL of 20 mM ATP (2 mM final...
concentration) was added to each protein or blank well to initiate ATPase activity. The plate was incubated in a 37°C water bath for 20 min, after which the reaction was stopped by the addition of 100 μL of stop reagent (3% (w/v) ascorbic acid, 0.5% (w/v) ammonium molybdate, 6% (w/v) SDS in 0.5 M HCl). After another 6 min, 100 μL of developing reagent (2% (w/v) sodium citrate, 2% (w/v) sodium arsenite, 2% (w/v) acetic acid) was added to each well. The colour was allowed to develop for 20 min and the absorbance of each well was measured at 750 nm. The A_{750} of the blank samples were subtracted from each sample value prior to calculating ATPase activity. All standard curve, blank, and protein samples were measured in triplicate.

To measure the effect of certain drugs on Pgp and Pgp-AzTMR ATPase activity at different drug concentrations, a 14-point ATPase profile was generated using a slightly modified 96-well plate assay. Drugs were serially diluted in DMSO to produce ~15 μL of drug at each concentration, ranging from 3 μM to 10 mM. Protein samples were diluted up to 88 μL in each well with 2 mM CHAPS/20 mM HEPES buffer, and 2 μL of drug at each concentration was added. To initiate the reaction, 10 μL of 20 mM ATP was added to each protein and control well. A 100% activity control, containing only protein and ATP, and a DMSO control, with 2 μL of DMSO in addition to protein and ATP were also tested. A single blank sample was made at each drug concentration and contained 88 μL of buffer, 2 μL of drug and 10 μL of 20 mM ATP. The plates were treated as above and all protein samples, the 100% and DMSO controls were assayed in triplicate. The absorbance values of each of the 14 blank samples were collectively averaged and this blank average was subtracted from each of the other samples prior to calculating the ATPase activity. The ATPase activity of the sample at each drug concentration was
divided by that of the DMSO control to obtain the % ATPase activity. The protein concentration in each well was appropriately diluted beforehand to ensure that the $A_{750}$ of the DMSO control sample was always approximately equal to the $A_{750}$ of the sixth point (10 nmol) in the $P_i$ standard curve to ensure reproducibility.

2.4 PREPARATION AND CHARACTERIZATION OF Pgp-AzTMR

All fluorescence measurements were carried out on a PTI QuantaMaster QM-8/2005 with Peltier temperature control, or a PTI QuantaMaster C-61 steady-state fluorimeter, at a constant temperature of 22°C, with excitation and emission slits set to 2 nm and 4 nm, respectively, unless otherwise stated.

2.4.1 Crosslinking of AzTMR to Pgp

Preparation of the Pgp-AzTMR adduct involved crosslinking AzTMR to ConA purified Pgp in a Stratalinker 1800 UV crosslinker with 365 nm bulbs (Stratagene, La Jolla, CA). A total reaction volume of 550 μL was prepared in a 6 dram acid-washed liquid scintillation vial (28 × 70 mm) on ice, containing 75-90 μg of purified Pgp in 2 mM CHAPS/20 mM HEPES buffer, 5 mM DTE, 2 mM ATP and 100 μM AzTMR in DMSO. The mixture was incubated on ice (in the dark) for 10 min, crosslinked in the Stratalinker 1800 for 20 min and incubated at 4°C for 10 min. The control, Pgp-mock, was prepared the same way, except the reaction mixture did not contain AzTMR and the volume was made up with buffer. The crosslinked and control samples were each loaded on to an Econo10-DG gel filtration column (1 × 6 cm) which had been pre-equilibrated with 2 mM CHAPS/20 mM HEPES buffer, and eluted with the same buffer. Fractions of 20 drops were collected and analyzed for absorbance at 280 nm, protein content, and
ATPase activity. Typically, one high protein concentration fraction (50 μg/mL) and one low concentration fraction of ~0.5 mL were collected, which were either pooled, or only the high concentration fraction was used, depending on the experiment.

2.4.2 Optimization of crosslinking conditions

The optimum conditions for crosslinking AzTMR to Pgp were determined by measuring the effect on Pgp-AzTMR formation of different initial AzTMR concentrations, time of UV exposure, and the presence of DTE or ATP. Samples were prepared as above and the UV exposure time was set at 0, 5, 10, 20, or 30 min. Controls were also performed where the samples were incubated for 20 min in the dark on ice and at room temperature. In addition, the optimal initial concentration of AzTMR was determined by crosslinking in the presence of 0, 10, 50, 100, 200, or 300 μM AzTMR. In a separate experiment, samples with and without 5 mM DTE or 2 mM ATP were crosslinked to determine the effect of both agents on labelling outcome. In all cases, the ATPase activity (relative to Pgp-mock), intrinsic Trp fluorescence (290 nm/330 nm), AzTMR fluorescence (545 nm/579 nm), and labelling stoichiometry were monitored to assess the outcome of the different sets of conditions.

2.4.3 Stoichiometry of AzTMR linkage

The stoichiometry of AzTMR covalently bound to Pgp was determined by interpolating the AzTMR concentration in Pgp-AzTMR samples from an AzTMR standard curve. Samples of AzTMR (0, 0.1, 0.25, 0.75 and 1.0 μM) were made in 2 mM CHAPS/20 mM HEPES buffer and subjected to the same treatment as Pgp-AzTMR samples (10 min on ice, 20 min UV treatment, 10 min on ice). The fluorescence of AzTMR at 579 nm was measured following excitation at 545 nm for the standard and
Pgp-AzTMR samples. Linear regression (Sigmaplot, Systat Software, Chicago, IL) of the plotted standard points allowed calculation of the AzTMR concentration in the Pgp-AzTMR samples. This was compared to the molar protein concentration (determined by Bradford assay), to estimate the stoichiometry of labelling.

2.4.4 Fluorescence properties of Pgp-AzTMR drug adduct

The excitation ($\lambda_{em} = 579$ nm) and emission spectra ($\lambda_{ex} = 545$ nm) of Pgp-AzTMR were each recorded 3 times and averaged. The spectra were automatically corrected by the instrument for lamp intensity variations. The background buffer spectra were subtracted and the spectra were normalized.

2.4.5 Polarity of the AzTMR binding site in Pgp

The polarity of the binding site of AzTMR within Pgp was characterized according to the Lippert equation, which relates the general solvent effects to the Stokes shift for a fluorophore in a particular environment (76;139):

$$\bar{\nu}_A - \bar{\nu}_F = \frac{2}{\hbar c} \left( \frac{\varepsilon - 1}{2 \varepsilon - 1} - \frac{n^2 - 1}{2n^2 + 1} \right) \left( \mu_E - \mu_G \right)^2 + \text{constant}$$

where $\bar{\nu}_A$ and $\bar{\nu}_F$ are the frequency (in cm$^{-1}$) of the absorption and emission maxima (Stokes shift), respectively, $\hbar$ is Planck’s constant ($6.626 \times 10^{-27}$ J·s), $c$ is the speed of light ($2.9979 \times 10^{10}$ cm/s), $\varepsilon$ is the dielectric constant, $n$ is the refractive index of the medium, $a$ (in cm) is the radius of cavity in which the fluorophore resides, and $\mu_E$ and $\mu_G$ are the dipole moments of the ground and excited states, respectively. The Lippert equation can be rearranged to a simpler form:

$$\bar{\nu}_A - \bar{\nu}_F = m \Delta f + \text{constant}$$
so that $\bar{\nu}_A - \bar{\nu}_F$ can be plotted against the orientation polarizability, $\Delta f$, and give a slope, $m$, which is known as the solvent sensitivity of the fluorophore. The orientation polarizability was calculated for each solvent according to:

$$\Delta f = \left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)$$

and is directly related to both the dielectric constant and refractive index of each environment. The Stokes shift of AzTMR solutions (0.3 μM) made in chloroform, methylene chloride, dimethylformamide (DMF), DMSO, 2-propanol, methanol and buffer was measured (in cm$^{-1}$) and plotted against the calculated $\Delta f$ value for each solvent. Linear regression of the data provided a Lippert plot from which the $\Delta f$ was calculated for AzTMR based on the measured Stokes shift of Pgp-AzTMR.

2.5 Determination of Pgp Drug Binding Affinity

2.5.1 Pgp intrinsic Trp fluorescence quenching

The binding of various drug substrates to Pgp and Pgp-AzTMR was determined by measuring the saturable, concentration-dependent quenching of the intrinsic Trp fluorescence of Pgp. A 250 μL sample of Pgp (50 μg/mL) in 2 mM CHAPS/20 mM HEPES buffer was added to a quartz microcuvette (0.5 cm path length, 1 mL total volume) and allowed to equilibrate for 5 min in the fluorimeter. Drug aliquots (dissolved in DMSO) of 0.5 μL were added, then the sample was mixed 20 times and allowed to equilibrate for 40 s. The steady-state Trp fluorescence of Pgp was measured by excitation at 290 nm, to avoid interference from Tyr fluorescence, and the emission was recorded at 330 nm for 10 s. The final concentration of DMSO was never more than 2.5% and control titrations with DMSO alone were performed to ensure the solvent was not
affecting Trp fluorescence. To correct for non-specific quenching of Trp fluorescence, an identical titration was performed with NATA, a soluble Trp analog. The starting concentration of NATA was typically 20 μg/mL, however, the concentration was adjusted for each experiment so the fluorescence emission intensity at 330 nm matched that of Pgp.

The experimentally measured fluorescence emission intensities \( (F_i) \) were corrected for the inner filter effect, light scattering and dilution (33;139) using the following equation:

\[
F_{icorr} = (F_i - B) \frac{(V_i/V_0) \left( (F_{0\text{NATA}} - B) \left( \frac{V_i}{V_0} \right) \right)}{(F_{i\text{NATA}} - B) \left( \frac{V_i}{V_0} \right)}
\]

where \( F_{icorr} \) is the corrected fluorescence intensity at any point (after the \( i \)th drug addition), \( B \) is the background fluorescence intensity of the buffer, \( V_0 \) is the initial volume, \( V_i \) is the volume at any point in the titration, \( F_{0\text{NATA}} \) is the initial uncorrected NATA fluorescence and \( F_{i\text{NATA}} \) is the NATA fluorescence after the \( i \)th addition. Thus, the dilution factor, \( V_i/V_0 \), and inner filter factor, \( [(F_{0\text{NATA}}-B)(V_i/V_0)]/[(F_{i\text{NATA}}-B)(V_i/V_0)] \), are both accounted for in calculating \( F_{icorr} \). The change in fluorescence, \( \Delta F \), is calculated as \( F_{icorr}-F_0 \), where \( F_0 \) is the initial fluorescence intensity, for the \( i \)th drug addition, plotted against the concentration of drug, and fitted to the following hyperbolic equation:

\[
\left( \frac{\Delta F}{F_0 \times 100} \right) = \frac{\left( \frac{\Delta F_{\text{max}}}{F_0} \times 100 \right) \times [S]}{K_d + [S]}
\]
Non-linear regression using the Marquardt-Levenberg algorithm was used to extract the values of the dissociation constant $K_d$, and the maximal percent fluorescence quenching $(\Delta F_{\text{max}}/F_0 \times 100)$ from this equation.

The fluorescence quenching data can also be fitted to a quadratic binding equation for monophasic quenching using Sigmaplot. The hyperbolic and quadratic equations give similar results for drugs that bind to Pgp with low affinity, namely those for which $K_d > [\text{Pgp}]$. However, for high affinity ligands, where the $K_d$ and Pgp concentration are approximately equal, the quadratic equation gives more accurate results, as this method accounts for the changes in drug concentration over the course of the binding experiment. For most drugs used, however, $K_d > [\text{Pgp}]$ and the hyperbolic equation is sufficient.

The site occupancy, $\theta$, was calculated for some drugs based on the derived $K_d$ at each drug concentration according to the following equation:

$$\theta_i = \frac{[\text{drug}]_i}{[\text{drug}]_i + K_d}$$

2.5.2 Enhanced H33342 and H33258 fluorescence on Pgp binding

The fluorescence emission of H33342 and H33258 was enhanced upon binding to Pgp, allowing quantification of the fluorescence enhancement and determination of binding affinity. Titrations were performed in a similar manner to those used to determine $K_d$ by intrinsic Trp quenching, with the exception that a buffer titration, instead of NATA, was used as a control. The steady-state fluorescence ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 354/480$ nm) was monitored and the data corrected for inner filter, light scattering and dilution, as previously described (139), using the following equation where $\text{P+L}$ and $\text{B+L}$ represent
protein plus ligand and buffer plus ligand, respectively:

$$\Delta F = \left[ (F_i - F_0) \left( \frac{V_i}{V_0} \right) \left( 10^{b(A_{\lambda_{ex}} + A_{\lambda_{em}})} \right) \right]_{P+L} - \left[ (F_i - F_0) \left( \frac{V_i}{V_0} \right) \left( 10^{b(A_{\lambda_{ex}} + A_{\lambda_{em}})} \right) \right]_{B+L}$$

where $F_0/F_i$ and $V_0/V_i$ are the initial and $i$th fluorescence intensity and volume, respectively, at any point in the titration, $b$ is the path length of the cuvette (in cm) and $A_{\lambda_{ex}}$ and $A_{\lambda_{em}}$ are the absorbance of the sample measured at the excitation and emission wavelengths, respectively. The corrected data were fitted to the same hyperbolic binding equation used for Trp quenching and the values of $K_d$ and $\Delta F_{\text{max}}$ were extracted by nonlinear regression of the data.

2.6 PGP TRANSPORT ASSAY

2.6.1 Functional reconstitution of Pgp

Pgp was reconstituted into DMPC proteoliposomes using a modification of a method previously developed in our lab (140). Approximately 45 μL of 100 mg/mL DMPC (4.5 mg total lipid) prepared in chloroform/methanol (4:1 v/v) was dried under nitrogen for 5 min and placed under vacuum for 45 min to remove the solvents. The white film was redissolved in 90 μL of 500 mM CHAPS/20 mM HEPES buffer, vortexed, and placed in a 37°C water bath for 5 min. Pgp (1 mL of ~250 μg/mL in 2 mM CHAPS/20 mM HEPES, 250 μg total protein) was added directly to the lipid solution and the mixture was passed through a 1 mL syringe with 26%G needle ~20 times. The mixture was gently mixed at 4°C for 45 min and then loaded on a 1 × 29 cm Sephadex G-50 gel filtration column which had been pre-equilibrated with 20 mM HEPES, to remove the CHAPS detergent. The sample was eluted with 20 mM HEPES and ~20 fractions (25 drops) were collected and analyzed for turbidity (absorbance at 280 nm), protein content
(Bradford assay) and ATPase activity. The three most turbid fractions were pooled to give a final volume of ~2 mL, with a final protein concentration of 108 μg/mL, an estimated lipid concentration of ~1.94 mg/mL, and lipid:protein ratio of 18:1 (w/w).

For the reconstitution of Pgp-AzTMR, the protein was first concentrated from its initial concentration of 50 μg/mL to a desired 250 μg/mL. Several batches of Pgp-AzTMR were prepared as described above (section 2.4.1) and the column fractions were concentrated using Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA). Briefly, three filter devices were used simultaneously with 500 μL of Pgp-AzTMR, and a spin time of 9 min at 4°C and 14,000g. The filters were continuously loaded with fresh aliquots of protein, and the concentrate from each was pooled to give a final volume of ~1 mL of ~250 μg/mL Pgp-AzTMR. Concentration of Pgp-AzTMR did not affect the ATPase activity of the protein, and the reconstitution was carried out as with Pgp, but a separate Sephadex G-50 column was used only for Pgp-AzTMR samples. The final protein concentration in Pgp-AzTMR proteoliposomes was ~85 μg/mL.

2.6.2 Real-time fluorescence transport assay

All fluorescence measurements for transport using DMPC proteoliposomes were carried out at 26°C. The proteoliposomes were diluted by adding 100 μL of 20 mM HEPES buffer pH 7.4 to 100 μL proteoliposomes and 10 μL of 125 μM H33342 (5.95 μM final H33342 concentration) and incubated for 10 min at 26°C (final Pgp concentration = 51.2 μg/mL, final Pgp-AzTMR concentration = 40.5 μg/mL and lipid concentration = 142 μg/mL). To measure transport, 200 μL of the sample was added to a quartz microcuvette (0.5 cm path length, 1 mL total volume), excited at 355 nm and continuously monitored for H33342 emission at 450 nm. After a 150 s equilibration, 50
μL of ATP plus an ATP regenerating system (5 mM ATP, 11.5 mg/mL creatine phosphate, 0.3 mg/mL creatine kinase in 20 mM HEPES pH 7.4) was added and the solution mixed 10 times (final ATP concentration = 1 mM, final H33342 concentration = 4.76 μM). Fluorescence monitoring was continued for a total of 10 min and the slope of the line for the first 10-20 s of transport was used to determine the initial transport rate (μM/min). A vanadate control, in which 5 μL of 5 mM sodium ortho-vanadate was added at the proteoliposome dilution stage (final vanadate concentration = 93 μM), was also analyzed to ensure that fluorescence changes did not result from dynamic repartitioning of the drug, and the vanadate spectrum subtracted from those without vanadate prior to calculating the slope. The sodium ortho-vanadate solution was heated at 100°C for 10 min prior to its addition to the proteoliposomes.
Chapter 3

CROSSLINKING OF AZTMR TO PURIFIED PGP
3.1 Introduction

Biochemical studies have revealed much useful information about the interaction of drugs with Pgp. The binding of drugs likely occurs by an induced-fit mechanism, whereby the drug molecule first enters the large binding pocket and then reorients in a specific sub-site to allow the most favourable interactions with the polypeptide. Drugs are thought to gain access to Pgp from the cytoplasmic leaflet of the membrane (76;77;141) and bind at the interface of the two TMDs, with residues contributed from both halves (142).

A number of studies have been completed by Loo and Clarke, who used covalent linkage of maleimide and MTS-drug derivatives to Pgp engineered with specific Cys mutations (55;80;91;143) to determine drug binding sites and measure changes in ATPase activity. However, none of these studies determined the binding affinity of unmodified and modified Pgp for individual drugs. Moreover, the Pgp used in these experiments had very low ATPase activity and the labelling sites were non-physiological. The conclusions from these experiments should be taken lightly due to the low basal ATPase activity, as they are probably under-estimations of the true activity (124).

The bacterial soluble transcriptional repressor, QacR, was crystallized in the presence of several different drugs (144), and some crystal structures showed two drugs bound at the same time in its large drug-binding pocket. In addition, the recent X-ray crystal structure of Pgp confirmed that it is also possible for multidrug efflux pumps to simultaneously interact with more than one drug (40). Pgp was crystallized in the absence and presence of the cyclic peptide inhibitor QZ59-RRR and its stereoisomer QZ59-SSS. The structure with QZ59-RRR was found to have one molecule of the peptide bound at a
sub-site near the middle of the large drug-binding pocket. Surprisingly, the structure with QZ59-SSS contained two bound molecules of peptide, found in unique binding sites above and below that of its stereoisomer. While there are doubts about the validity of this structure physiologically, it does provide the first structural description of two substrate molecules bound to Pgp at the same time.

Fluorescence spectroscopy and changes in ATPase activity have been extremely useful tools in studying the interaction of drugs with Pgp. They have allowed for the determination of drug binding affinity (K_d) and even demonstrated the ability of two drugs to bind simultaneously to Pgp (54;55;77;141). In one study carried out in the Sharom lab, the binding of rhodamine 123 and LDS-751 showed a reciprocal negative interaction, with each drug reducing the binding affinity of the other by 5-fold (54). The two substrates in this case had complementary fluorescence properties, and because the free and bound drugs could not be separated, a complex global fitting analysis had to be used to determine the K_d values. This specialized approach does not allow for the study of simultaneous binding of a range of other Pgp substrates. Therefore, a new and more generally applicable approach was needed to look at simultaneous binding of two drugs.

One objective of this thesis was to explore and measure the binding of a second drug to Pgp with another drug covalently linked to the binding pocket, to understand how binding of the first drug molecule affects the subsequent binding of the second drug molecule. To achieve this, we sought to covalently link AzTMR, a fluorescent azido-derivative of the Pgp substrate TMR, to the drug-binding pocket of Pgp and analyze the biochemical and fluorescence properties of the drug adduct. The hope is that the drug would crosslink to the site within Pgp where it normally binds, which offers a significant
advantage over probes which link non-specifically or to engineered cysteine residues. The azido group is activated by UV light and reacts with protein side chains via a free radical mechanism. Thus, it was necessary to thoroughly study the adduct after crosslinking to ensure the highly reactive AzTMR did not cause any negative effects on Pgp. By labelling Pgp in this manner, unbound probe can be removed from Pgp-AzTMR by chromatography so the equilibrium between free and bound drug need not be considered. The Pgp-drug adduct can then be used to determine how the presence of covalently-bound TMR affects binding of a second drug molecule.

3.2 COVALENT CROSSSLINKING OF AZTMR TO PGP

To establish crosslinking of AzTMR to purified Pgp in CHAPS detergent, AzTMR was activated by UV irradiation in a Stratalinker 1800 UV crosslinker. ATP was added to the reaction mixture to protect the ATP binding site during UV activation. DTE was also added to the mixture in order to preserve as much ATPase activity as possible. The reaction mixture, consisting of Pgp, ATP, DTE, AzTMR and CHAPS buffer, was first incubated on ice in the dark for 10 min, followed by 20 min of crosslinking with 365 nm UV light. The crosslinked mixture was then incubated at 4°C for another 10 min and finally separated by gel filtration chromatography. A Pgp-mock sample (Pgp, ATP, DTE and CHAPS buffer) was prepared under the same conditions as Pgp-AzTMR to gauge whether the crosslinking procedure had any effect on the catalytic activity of Pgp. The ATPase activity of Pgp-mock was similar to that of Pgp, indicating that UV treatment alone did not affect ATPase activity. The ATPase activity of the Pgp-AzTMR drug adduct was approximately 55-75% of that of native Pgp; a similar decrease in ATPase activity was seen for unlabelled Pgp in the presence of 100 μM free AzTMR.
Upon excitation at 545 nm, the fluorescence emission maximum of AzTMR in buffer was 581 nm, however, once crosslinked to Pgp, a 3 nm blue shift was seen (Figure 3.1A). A blue shift is indicative of movement of AzTMR into a more hydrophobic environment, however, it is a relatively small shift. The intrinsic Trp fluorescence of Pgp decreased 25-50% upon formation of the Pgp-AzTMR adduct (Figure 3.1B). A decrease in Pgp Trp fluorescence is typically seen for certain drugs that result in concentration-dependent quenching following excitation at 290 nm. Many of these drugs have a higher maximal excitation and emission wavelength than Pgp intrinsic Trp fluorescence, such as AzTMR does (68).

3.3 OPTIMIZATION OF CROSSLINKING CONDITIONS

Prior to undertaking any experiments with Pgp-AzTMR, it was necessary to determine the best crosslinking conditions to ensure optimal fluorescence, ATPase activity and stoichiometry of the Pgp drug adduct. To this end, crosslinking experiments were carried out in which the UV exposure time was varied from 0 to 30 min and with an initial concentration of AzTMR ranging from 0-300 μM. In addition, the effects of DTE and ATP in this process were quantified. To measure the effectiveness of each set of conditions, AzTMR and Pgp intrinsic Trp emission fluorescence intensity were determined, along with the resultant Pgp-AzTMR ATPase activity.

To determine the optimum length of UV exposure, a crosslinking timecourse experiment was completed with exposure times to 365 nm UV light of 0, 5, 10, 20 and 30 min. The 0 min (non-irradiated) sample was incubated at room temperature for 20 min with exposure to room lighting. Pgp-mock was exposed to UV for 20 min in the absence
Figure 3.1  Fluorescence properties of Pgp and Pgp-AzTMR

Normalized fluorescence emission scans of Pgp and Pgp-AzTMR. Panel A shows the blue shift in AzTMR fluorescence ($\lambda_{ex} = 545$ nm) after formation of the Pgp-AzTMR adduct. The changes in intrinsic Trp fluorescence ($\lambda_{ex} = 290$ nm) are shown in Panel B for Pgp-AzTMR adduct formation, and for Pgp exposed to UV light in the absence of drug (Pgp-mock). The emission scans were corrected for protein concentration (Au = arbitrary units).
of AzTMR as a control for Pgp exposure to UV light. In addition, a Pgp sample was incubated with AzTMR for 20 min in the dark (Pgp-dark) to observe the effects of no light exposure. There was a small increase in bound AzTMR fluorescence compared to Pgp-mock (Figure 3.2B) for the 0 min sample, along with a slight decrease in Trp fluorescence relative to Pgp-mock (Figure 3.2A). This indicates that the exposure to room lighting did result in a slight crosslinking of AzTMR to Pgp, and the bound AzTMR quenched Trp fluorescence as expected. The Pgp-dark sample, however, showed no bound AzTMR fluorescence, indicating that incubation in the absence of UV irradiation does not result in activation and crosslinking of AzTMR to Pgp, and they can subsequently be separated from each other. Pgp-mock retained Trp fluorescence intensity and ATPase activity (Figure 3.2C), suggesting that 365 nm UV exposure did not damage the protein. The ATPase activity of the 0 min sample was similar to that of Pgp-mock, while that of Pgp-dark was slightly lower.

Over the range of the UV irradiation times used, the Trp fluorescence intensity of Pgp did not change to a significant degree. The AzTMR fluorescence, being a measure of labelling, increased with time up to a maximum level after 10 min of UV exposure. The ATPase activity decreased with increasing UV irradiation time, and leveled off ~30% lower than that of Pgp-mock. The data suggest that 10, 20, or 30 min of 365 nm UV exposure would be sufficient for optimum labelling and ATPase activity of Pgp-AzTMR, however, to ensure no under- or over-exposure (which can lead to photobleaching), 20 min was chosen as the optimal time for all subsequent experiments.

In order to determine the optimum initial concentration of AzTMR, the concentration was varied from 0-300 μM and the resulting Pgp-AzTMR monitored for
Figure 3.2  Crosslinking timecourse of AzTMR to Pgp

Intrinsic Trp fluorescence (Panel A), bound AzTMR fluorescence (Panel B), and ATPase activity (Panel C) measured at each time of UV crosslinking in the presence of 100 μM AzTMR. The 0 min sample was exposed for 20 min to room lighting. Pgp-mock (Δ) was irradiated for 20 min in the absence of AzTMR, Pgp-dark (×) was left for 20 min in the dark. All other samples were exposed to UV light in the Stratalinker (●). Fluorescence measurements were corrected for protein concentration and Pgp-mock was used as the 100% ATPase activity benchmark. All values are the average of 3 experiments ± SE.
Trp and bound AzTMR fluorescence and ATPase activity. The 0 μM sample was effectively equivalent to Pgp-mock and had the highest Trp fluorescence and ATPase activity, with no bound AzTMR fluorescence. The AzTMR fluorescence intensity increased with concentration to a maximum at 300 μM (Figure 3.3B), and the Trp fluorescence decreased as expected, since increased AzTMR crosslinking led to more internal Trp quenching (Figure 3.3A). The Pgp ATPase activity decreased as the AzTMR concentration increased, and was about 40% lower at 100 μM (Figure 3.3C). To maximize Trp emission (important for binding experiments) as well as AzTMR labelling and ATPase activity, a concentration of 100 μM AzTMR was chosen for crosslinking in further experiments.

DTE has been observed in our lab to protect Pgp ATPase activity, while the presence of ATP is thought to protect Pgp function (likely by maintaining its native conformation), therefore, the addition of both of these agents was tested for their effect on AzTMR crosslinking. The fluorescence emission of bound AzTMR was similar for samples containing 2 mM ATP in either the presence or absence of 5 mM DTE, and significantly higher than those containing no ATP (Figure 3.4B). This suggests that ATP is required for optimal crosslinking of AzTMR to Pgp, while DTE is not. The effect of ATP and DTE on Pgp-AzTMR ATPase activity was significant (Figure 3.4C); samples lacking both agents showed markedly decreased activity upon crosslinking. Both samples containing DTE, with and without ATP, had the highest ATPase activity following crosslinking. All samples displayed a ~50% reduction in Pgp Trp fluorescence following crosslinking (Figure 3.4A). In the samples containing ATP, this decrease was likely due to quenching of Trp fluorescence by linked AzTMR. Based on these results, it appears
Figure 3.3  Effect of different initial AzTMR concentrations on crosslinking

The concentration of AzTMR added to the reaction mixture was varied to optimize Pgp-AzTMR adduct formation. The intrinsic Trp fluorescence (Panel A), AzTMR fluorescence (Panel B) and ATPase activity (Panel C) are shown for each concentration. The 0 μM sample is effectively the same as Pgp-mock. Fluorescence measurements were corrected for protein concentration and Pgp-mock was used as the 100% ATPase activity benchmark. All values are the average of 3 experiments ± SE.
Figure 3.4  Effect of ATP and DTE on Pgp-AzTMR crosslinking

The intrinsic Trp fluorescence (Panel A), bound AzTMR fluorescence (Panel B), and ATPase activity (Panel C) were measured following incubation with 2 mM ATP/5 mM DTE (+) or without ATP/DTE (-), followed by UV exposure for 20 min. Fluorescence measurements were corrected for protein concentration and Pgp-mock was used as the 100% ATPase activity benchmark. All values are the average of 3 experiments ± SE.
that both ATP and DTE are beneficial to ensure maximum AzTMR labelling while minimizing the negative effects of UV exposure on Pgp ATPase activity. Therefore, 2 mM ATP and 5 mM DTE were included in all subsequent crosslinking experiments.

### 3.4 Stoichiometry of Covalent Linkage of AzTMR to Pgp

It was necessary to determine the drug labelling stoichiometry of AzTMR to Pgp in order to assess the outcome of various crosslinking conditions. A standard curve of AzTMR absorbance at 545 nm was generated at concentrations from 0-25 μM. However, when the AzTMR concentration was interpolated based on Pgp-AzTMR absorbance at 545 nm, the labelling ratio varied from 6 to 48, depending on the starting concentration of AzTMR used. It seems likely that the absorbance standard curve was overestimating the AzTMR concentration in the Pgp-AzTMR samples because the measured absorbance signal was very low. As a result, a standard curve of AzTMR fluorescence emission at 579 nm (λ<sub>ex</sub> = 545 nm) was generated for AzTMR concentrations from 0-1 μM (Figure 3.5A). These standards were treated in the same way as Pgp-AzTMR and Pgp-mock (including UV exposure). The AzTMR standard curve was linear (Figure 3.5A) and allowed estimation of the AzTMR concentration of the Pgp-AzTMR solutions. The molar stoichiometry was determined by comparison with the molar protein concentration for each of the tested conditions of Pgp labelling. The stoichiometry increased with the time of UV exposure up to a maximum of just over 1 at 20-30 min. The stoichiometry also appeared to increase with AzTMR concentration, but did not appear to reach a maximum, which is likely due to the inability of the gel filtration column to sufficiently separate Pgp-AzTMR from unlabelled AzTMR at very high concentrations. ATP is an absolute requirement for efficient AzTMR labelling; in its absence labelling was found to be
Figure 3.5  Stoichiometry of AzTMR labelling of Pgp

Standard curve of free AzTMR (Panel A) used in stoichiometry determination. The standards in 2 mM CHAPS/HEPES buffer were exposed to UV light for 20 min. The fluorescence was measured at 579 nm (λ<sub>ex</sub> = 545 nm) and the standard curve generated by linear regression of the data. The molar stoichiometry of drug labelling was determined at each time in the crosslinking experiment for Pgp (●) and for Pgp-mock (Δ) (Panel B). All values are the average of 3 experiments ± SE and where not visible, the error bars are contained within the symbols.
Panel C shows the AzTMR drug labelling stoichiometry at each of the initial AzTMR concentrations tested. In Panel D, the effects of 2 mM ATP and 5 mM DTE on AzTMR labelling stoichiometry were determined. All values are the average of 3 experiments ± SE and where not visible, the error bars are contained within the symbols.
20-40% (Figure 3.5D). In contrast, DTE did not appear to be required for stoichiometric labelling, but served an important role in maintaining ATPase activity. Under optimized conditions for crosslinking, the molar stoichiometry of AzTMR labelling of Pgp was repeatedly found to be \( \sim 1 \).

3.5 DISCUSSION

The activation of AzTMR by UV light produces a highly reactive aryl azide intermediate which acts as a powerful nucleophile in a number of different reactions. It can react with hydrocarbons, amines, and carbon-carbon double bonds which are present in the amino acid side chains of proteins (145). The goal of this project was to covalently link a fluorescent probe, AzTMR, to its physiological binding site within the Pgp binding pocket. To ensure that this probe was not labelling Pgp in a non-specific way, the Pgp-AzTMR drug adduct was thoroughly characterized. The ATPase activity of the Pgp-AzTMR drug adduct decreased by 25-45% and the intrinsic Trp fluorescence by 25-50% (Figure 3.1B), both of which were consistent with what was observed for noncovalent binding of TMR. Furthermore, the fluorescence emission peak of AzTMR shifted to lower wavelengths by \( \sim 3 \) nm. This blue shift is consistent with the TMR fluorophore moving from an aqueous to a more non-polar environment, such as that found in the interior of proteins. Earlier studies on the drug-binding pocket of Pgp suggested that its environment resembles one with a polarity lower than that of chloroform (76). If the AzTMR probe were merely crosslinking to the protein surface in a non-specific way, no change would be expected in the emission spectrum of the fluorophore.
The goal of the timecourse experiment was to determine the optimum UV light exposure for crosslinking AzTMR to Pgp to achieve a molar labelling stoichiometry close to 1. A secondary aim was also to determine whether the changes observed in ATPase activity, Trp fluorescence and AzTMR fluorescence were due to UV exposure or formation of the drug adduct. For this reason, several control experiments were conducted, including a 0 minute sample (20 min at room temperature and exposure to room lighting), Pgp-dark (20 min in the dark) and Pgp-mock (UV-exposed sample with no AzTMR). The results indicated that AzTMR does not crosslink significantly to Pgp in the absence of UV irradiation (a small amount of crosslinking to Pgp occurred as a result of exposure to room lighting).

The concentration of AzTMR in the reaction mixture was an important parameter to optimize, as different concentrations resulted in different ATPase activity and apparent stoichiometry of the Pgp-AzTMR adduct. The K_d for AzTMR non-covalent binding to Pgp is 27 μM (Section 4.3.2), and the most effective concentrations in crosslinking were higher than this. The activated azido group is very reactive and, given the large molar excess of label, it might be surprising that only one molecule of AzTMR was found covalently linked to Pgp. A study with the nucleotide photoaffinity labels 2-azido-ATP and 8-azido-ATP showed that while both analogs are capable of binding to the catalytic site of Pgp, only the latter is able to covalently react after photoactivation (146). The authors suggested that Pgp had no suitable amino acid side chains adjacent to the photoactivated 2-position of 2-azido-ATP. This explanation is also applicable to the photoactivation of AzTMR; there may be limited availability of suitable reactive amino acid side chains in the immediate vicinity of the TMR binding sites. Therefore, although
more than one molecule of AzTMR may bind to Pgp, it is possible that only one binding site is labelled following photoactivation.

The role of ATP and DTE was also investigated as part of optimizing the AzTMR labelling efficiency to Pgp. The effect of adding 2 mM ATP was significant, with samples containing ATP showing 60-80% more AzTMR labelling than those without. The decrease in intrinsic Trp fluorescence on AzTMR labelling can likely be attributed to quenching by crosslinked AzTMR. The transport cycle of Pgp involves both drug and nucleotide binding, and one study found different conformations of the TMDs in different nucleotide-bound states of Pgp (50). It is thought that the substrate-binding site may be more accessible to drug in the presence of nucleotide through conformational changes transmitted from the NBDs to the TMDs. Thus ATP binding may help facilitate drug binding to Pgp, which explains why the presence of ATP resulted in more efficient crosslinking of AzTMR. Alternatively, ATP (as a substrate) may stabilize the native sandwich dimer conformation of Pgp.

Crosslinking AzTMR to Pgp resulted in a 25-45% decrease in the ATPase activity. Many substrates are found to either stimulate or inhibit ATPase activity, or both (103;104;121). The effect of free AzTMR on Pgp ATPase activity is concentration dependent; it was observed to slightly stimulate activity at very low concentrations and then inhibit activity to below basal levels at higher concentrations (Section 4.3.1). At saturating concentrations of AzTMR, Pgp ATPase activity is ~50% lower, which is consistent with the activity of Pgp-AzTMR after crosslinking. Recent models for the catalytic cycle of Pgp suggest that it can participate in either basal ATPase activity or drug-coupled activity (124). In the basal cycle, ATP is turned over at a constant rate until
the binding of substrate shifts the protein to the drug-coupled cycle. This binding is proposed to be dependent on the specific drug; the better it is at forming the transition state for the drug-coupled cycle, the faster it is transported. Covalent linking of AzTMR to Pgp would effectively move it into the drug-coupled cycle.
Chapter 4

BIOCHEMICAL CHARACTERIATION OF PGP-AZTMR
4.1 Introduction

The drug binding nature of Pgp has been actively studied in an effort to define a binding site (or sites) that can be targeted by inhibitors. The binding affinity of Pgp has been measured for a wide range of substrates, and the $K_d$ values range from the nM to μM scales (61). The X-ray structures of Pgp in the presence of cyclic peptide inhibitors indicate that the large binding pocket contains at least three, possibly more, distinct binding sub-sites. Pgp cannot possibly contain a unique binding site for each of its hundreds of substrates, however, its structures, along with those of other multidrug binding proteins (147), reveal how they are capable of poly-specific drug binding. The goal of this chapter was to characterize the sub-site where AzTMR is crosslinked within the binding pocket of Pgp.

The binding sites of Pgp for H33342 and LDS-751 have been mapped to the cytoplasmic leaflet of the membrane (76;77). The LDS-751 site has been described as having a nonpolar, hydrophobic environment (76), however, Clarke and coworkers have suggested that the binding pocket is accessible to water (92). The polarity of the environment for AzTMR covalently crosslinked to Pgp may be determined by measuring the Stokes shift of AzTMR in a variety of solvents and constructing a Lippert plot, which relates the Stokes shift to solvent polarity.

The transport cycle of Pgp involves coupling the processes of drug binding and translocation to the binding and hydrolysis of ATP. Drug binding to the TMDs induces conformational changes in Pgp that initiate the transport process (123). The ability of drugs to stimulate or inhibit Pgp ATPase activity is thus a direct consequence of drug binding and the resulting conformational changes. Any changes in ATPase activity could
indicate that binding of the second drug within the Pgp binding pocket is altered by the presence of linked AzTMR.

In this chapter, the binding affinity of Pgp and Pgp-AzTMR for various substrates (Figure 4.1) was determined, to provide some information as to where the compound is binding. If a drug is added that normally binds at the site occupied by the covalently linked TMR group, a decrease in binding affinity relative to native Pgp would be expected. Conversely, if the location of the linked AzTMR group in Pgp-AzTMR does not infringe on the binding site of the second drug, no change in binding affinity would be expected. Two functional Pgp drug transport sites, the H- and R-site, were identified based on their preference for transporting H33342 or R123, respectively (127). Further characterization of the Pgp binding pocket was undertaken by determining the binding affinity of Pgp-AzTMR for a selection of known R- and H-site substrates. If the fold-change in binding affinity of the R- and H-site drugs to Pgp-AzTMR relative to Pgp is different for each drug, then it would suggest that there is no simple R- and H-site relationship, but rather a large flexible binding pocket. However, if the R- and H-sites behave as originally proposed, binding of R-site drugs might be inhibited by covalent linkage of AzTMR, while H-site drugs might show little change in binding affinity.

In support of earlier thermodynamic and biochemical work, Chiba and coworkers have suggested that Pgp may contain two transport pathways (148). Using site-directed mutagenesis of key residues, they showed that Pgp contains two pseudosymmetric translocation pathways, with different drugs having a preference for one over the other. If Pgp-AzTMR retains its ability to transport either H- or R-site drugs, it could indicate that
Figure 4.1 Some Pgp substrates used in experiments with Pgp and Pgp-AzTMR

The binding affinity and ATPase activity profile of each of these compounds was determined for Pgp and Pgp-AzTMR. The rhodamine compounds are proposed to interact with the R-site, while H33342 and H33258 are proposed to interact with the H-site (127). LDS-751 is proposed to interact with the R-site (130), but transport data from our lab suggest that it might interact with both sites.
one or both of these pathways are still able to function with AzTMR bound to the R-site within the binding pocket.

4.2 Determining the polarity of the local environment of bound AzTMR in Pgp

For a number of fluorophores, their excitation and emission spectra are dependent on the polarity of their local environment. After crosslinking of AzTMR to Pgp, a small blue shift was seen in its emission spectrum (Figure 3.1). As a rule of a thumb, fluorophores experience a shift in their fluorescence emission spectra towards higher energies when moving to a more nonpolar, or hydrophobic, environment. However, because excitation spectra can also shift to higher or lower energies in different environments, the difference between absorption and emission maxima, or Stokes shift ($\nu_A-\nu_E$), is a more quantitative way of analyzing that change. The Stokes shift of AzTMR (in wavenumbers, cm$^{-1}$) was measured in a number of different solvents ranging from low to high polarity: chloroform, methylene chloride, DMF, DMSO, 2-propanol, methanol and aqueous buffer. These solvents were chosen based on the ability of AzTMR to dissolve in them at a concentration of 0.30 $\mu$M without any harsh conditions. The orientation polarizability ($\Delta f$) of each solvent was determined based on its refractive index and dielectric constant (Section 2.4.5) and used as a relative measure of that solvent’s polar or nonpolar nature. The measured Stokes shift of AzTMR in each solvent was plotted against solvent orientation polarizability in the Lippert plot (shown in Figure 4.2). Linear regression of the data gave the Lippert equation (m = 1444 cm$^{-1}$) for AzTMR in a variety of environments. The measured Stokes shift of AzTMR crosslinked to Pgp of 1028 cm$^{-1}$ was used to interpolate a polarizability of 0.2799 from the Lippert equation.
Figure 4.2  Lippert plot of AzTMR in solvents of varying polarity

The Stokes shift of AzTMR was measured in chloroform (1), methylene chloride (2), DMF (3), DMSO (4), propanol (5), methanol (6) and aqueous buffer (7). The measured Stokes shift of the AzTMR group in labelled Pgp is indicated on the graph at 1027.8 cm$^{-1}$. Data are the average of 2 independent experiments. Where not visible, error bars are contained within the symbols.
This value of $\Delta f$ was lower than those for both methanol (0.3086) and buffer (0.3199) and only slightly higher than for propanol (0.2762), suggesting that AzTMR did move to a more hydrophobic environment relative to buffer. It is interesting to note, however, that the AzTMR binding site is not as hydrophobic as that previously determined for LDS-751 (76), which explains the relatively small (3 nm) blue shift seen for Pgp-AzTMR.

4.3 Interaction of R-site Drugs with Pgp and Pgp-AzTMR

4.3.1 Effect on Pgp ATPase activity

The effect on Pgp and Pgp-AzTMR ATPase activity of several rhodamine dyes (R-site drugs) and LDS-751, a potential R-site drug (130), was determined over a range of drug concentrations. The rhodamines all contain the same aromatic core structure, with different types and degrees of substitution (Figure 4.1). It is not known whether it is the core structure or the substituents that are responsible for orienting the drug in a particular region of the binding pocket. It seems plausible that the substituents would have a role in providing specific contacts to the protein in addition to the $\pi-\pi$ interactions afforded by the aromatic ring structure. Substrates can inhibit, stimulate, or have a biphasic effect on Pgp ATPase activity, which could be a result of the particular binding location of that drug, and the effect that binding has on communication with the NBDs.

The ATPase profile for free AzTMR was determined for both native Pgp and Pgp-AzTMR (Figure 4.3 A). For Pgp, ATPase activity was stimulated ~20% at concentrations up to 5 $\mu$M and inhibited at higher concentrations, with inhibition reaching ~50% at 200 $\mu$M. Low concentrations of AzTMR were slightly stimulatory towards Pgp-AzTMR, however, it inhibited activity at much lower concentrations than for Pgp. The ATPase profiles with R101, R110, R123, R6G, and TMR generally showed lower stimulation
Figure 4.3 Effect of AzTMR on ATPase activity and binding of Pgp and Pgp-AzTMR

ATPase activity of Pgp and Pgp-AzTMR (relative to the DMSO control) with increasing concentrations of free AzTMR (Panel A), and quenching of Trp fluorescence by AzTMR (Panel B) for Pgp and Pgp-AzTMR. Data are the average of 3 determinations ± SE. Binding data were corrected for buffer background, inner filter effect and dilution.
levels at low concentrations for Pgp-AzTMR relative to Pgp, however, the overall shape of the curve and inhibition region were relatively unchanged (see Figure 4.4A for TMR). Suprisingly, the ATPase profiles of TMR-E and TMR-M were different from those of the other rhodamines. At the concentrations tested, both TMR-E and TMR-M stimulated activity, but did not show any inhibition at high concentration (see Figure 4.4B for TMR-E). For Pgp, LDS-751 was largely stimulatory or had little effect on ATPase activity up to 300 μM, but inhibited Pgp-AzTMR activity above 1 μM, much like free AzTMR.

4.3.2 Binding affinity of Pgp for R-site drugs

The binding affinities of Pgp and Pgp-AzTMR for the R-site drugs were determined by measuring the concentration-dependent saturable quenching of intrinsic Trp fluorescence (λ_ex/λ_em = 290/330 nm). The rhodamines are all fluorescent, however, they did not undergo a substantial enhancement or quenching of fluorescence upon binding to Pgp or Pgp-AzTMR. Initially, drug titrations were completed with Pgp-mock, however, once it became apparent that there was no significant difference in the binding affinity of Pgp compared to Pgp-mock, Pgp was used in all subsequent experiments. This is supported by the findings that UV exposure alone had a negligible impact on Pgp, and that Pgp-mock exhibited no changes in ATPase activity or fluorescence when compared to Pgp.

The binding affinities (measured as K_d) of Pgp and Pgp-AzTMR for free AzTMR were determined to be 26.3 μM and 78.9 μM, respectively (Figure 4.3B), representing a 3-fold decrease in the affinity of Pgp-AzTMR for a second molecule of AzTMR. The binding curve for several of the rhodamines rose faster and saturated at lower concentrations for Pgp relative to Pgp-AzTMR; a representative drug from this group was
Figure 4.4  Effect of TMR and TMR-E on ATPase activity of Pgp and Pgp-AzTMR

ATPase activity of labelled and unlabelled Pgp at concentrations from 0.6 μM to 300 μM TMR (Panel A) and TMR-E (Panel B). The DMSO control was used as the 100% activity control. Data are the average of 3 determinations ± SE.
TMR, whose binding plots are shown in Figure 4.5A. Similarly to AzTMR, the binding affinities of TMR, R123, R6G, R101 and R110 decreased by 3.9-, 1.1-, 2.4-, 1.7- and 1.8-fold, respectively, for Pgp-AzTMR compared to Pgp (Table 4.1). Like the majority of rhodamines, the binding affinity of the Pgp-AzTMR adduct for LDS-751 decreased 1.7-fold relative to unlabelled Pgp. Surprisingly, however, TMR-E (Figure 4.5B) and TMR-M were bound with higher affinity by Pgp-AzTMR compared to Pgp, giving a 0.5- and 0.7-fold change in $K_d$.

Table 4.1  Binding affinity of Pgp and Pgp-AzTMR for R-site drugs

<table>
<thead>
<tr>
<th>drug</th>
<th>$K_d$ ($\mu$M)</th>
<th>$\Delta F_{max}$ (%)</th>
<th>$K_d$ ($\mu$M)</th>
<th>$\Delta F_{max}$ (%)</th>
<th>relative $K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R123</td>
<td>31.6 ± 4.0</td>
<td>53.7 ± 2.9</td>
<td>33.5 ± 2.2</td>
<td>38.5 ± 0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>AzTMR</td>
<td>26.3 ± 1.3</td>
<td>69.1 ± 1.4</td>
<td>78.9 ± 9.1</td>
<td>81.9 ± 3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>R110</td>
<td>7.1 ± 0.9</td>
<td>28.3 ± 1.3</td>
<td>13.0 ± 1.4</td>
<td>26.9 ± 1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>R101</td>
<td>6.6 ± 0.2</td>
<td>62.1 ± 0.6</td>
<td>11.3 ± 0.9</td>
<td>65.4 ± 1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>TMR</td>
<td>4.4 ± 0.4</td>
<td>62.7 ± 2.0</td>
<td>17.0 ± 2.1</td>
<td>69.9 ± 2.9</td>
<td>3.9</td>
</tr>
<tr>
<td>R6G</td>
<td>3.1 ± 0.4</td>
<td>33.5 ± 1.7</td>
<td>7.4 ± 0.7</td>
<td>40.0 ± 1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>TMR-E</td>
<td>3.0 ± 0.3</td>
<td>30.2 ± 1.4</td>
<td>1.5 ± 0.2</td>
<td>30.7 ± 1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>LDS-751</td>
<td>2.2 ± 0.2</td>
<td>52.3 ± 1.6</td>
<td>3.8 ± 0.3</td>
<td>59.7 ± 1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>TMR-M</td>
<td>2.1 ± 0.2</td>
<td>31.9 ± 1.0</td>
<td>1.5 ± 0.4</td>
<td>30.5 ± 1.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

1 $K_d$ values were the mean of at least 3 independent experiments (± SE).

2 fold-change = ($K_d$)Pgp-AzTMR/($K_d$)Pgp

In general, the maximal change in Trp fluorescence intensity ($\Delta F_{max}$) for each drug was relatively similar for AzTMR-labelled and unlabelled Pgp (Table 4.1). However, the $\Delta F_{max}$ varied greatly from 30-70% for the range of compounds used, and no trends between $K_d$ and $\Delta F_{max}$ were evident, consistent with prior observations (32). Drugs
Figure 4.5  TMR and TMR-E binding to Pgp and Pgp-AzTMR

Quenching of Trp fluorescence by TMR (Panel A) and TMR-E (Panel B) for Pgp and Pgp-AzTMR. Data are the average of 3 independent experiments ± SE. Data were corrected for buffer background, inner filter effect and dilution.
showing a large $\Delta F_{\text{max}}$ are typically able to accept energy from excited Trp residues or make more $\pi-\pi$ stacking interactions with aromatic residues of the protein (62).

4.4 **INTERACTION OF H-SITE DRUGS WITH PGP AND PGP-AZTMR**

4.4.1 **Effects on Pgp ATPase activity**

The two H-site drugs used in this study were H33342 and H33258, which contain the same basic aromatic ring backbone but differ in a single substituent; H33342 contains an *ortho* alcohol group, while H33258 contains an ethyl ether group (Figure 4.1). The effect of H33342 and H33258 on Pgp and Pgp-AzTMR ATPase activity was determined at concentrations up to 300 $\mu$M; a representative plot for H33342 is shown in Figure 4.6. Both H33342 and H33258 showed biphasic effects on Pgp ATPase activity, stimulating it slightly up to 0.7 $\mu$M and 9.0 $\mu$M, respectively, and inhibiting it at concentrations higher than those thresholds. Moreover, the ATPase activity profiles for both drugs with Pgp-AzTMR were nearly superimposable on those with Pgp, suggesting that H-site stimulated/inhibited ATPase activity was little changed from Pgp to Pgp-AzTMR.

4.4.2 **Binding affinity of Pgp for H-site drugs**

When titrating the H-site drugs (H33342 and H33258) with Pgp, a concentration-dependent enhancement of Hoechst fluorescence emission was observed for both compounds. Moreover, the fluorescence spectra of the Hoechst compounds did not overlap with those of either AzTMR or Trp as did those of the rhodamines. Therefore, the binding affinity of Pgp and Pgp-AzTMR for H33342 and H33258 was determined by measuring both the quenching of intrinsic Trp fluorescence ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 290/330$ nm) and the enhancement of Hoechst fluorescence ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 354/480$ nm). Both sets of data were
Figure 4.6  Effect of H33342 on ATPase activity of Pgp and Pgp-AzTMR

The ATPase activity of Pgp and Pgp-AzTMR was measured in the presence of various concentrations of H33342 ranging from 0.6 μM to 300 μM. Each data point is the average of triplicate measurements ± SE. The DMSO control was used as the 100% activity control.
fitted to a hyperbolic monophasic binding equation and $K_d$ and $\Delta F_{\text{max}}$ values were extracted (Table 4.2). Representative plots for Trp quenching by H33342 are shown in Figure 4.7A, and are little changed from Pgp to Pgp-AzTMR. Indeed, both H33342 and H33258 bind to Pgp and Pgp-AzTMR with nearly identical $K_d$ values when determined by Trp quenching (Table 4.2). The binding affinities for H33342 and H33258 determined by Hoechst fluorescence enhancement were slightly higher for Pgp-AzTMR, with 0.7- and 0.9-fold changes relative to Pgp, respectively (Figure 4.7B). In general, the $K_d$ values obtained by Hoechst fluorescence enhancement were higher than those obtained by Trp quenching, which is likely due to the different ways the data were obtained and corrected. However, within each data set, it appears that both H-site drugs bind to Pgp-AzTMR with equal or higher affinity compared to Pgp.

| Table 4.2 Binding affinity of Pgp and Pgp-AzTMR for Hoechst compounds |
|------------------|------------------|------------------|
|                  | Pgp              | Pgp-AzTMR        | relative $K_d$ |
| **Trp quenching**|                  |                  |                |
| drug             | $K_d$ (μM)       | $\Delta F_{\text{max}}$ (%) | $K_d$ (μM)       | $\Delta F_{\text{max}}$ (%) | (fold change)$^1$ |
| H33342           | 3.1 ± 0.3        | 64.5 ± 1.8       | 3.2 ± 0.4       | 66.1 ± 3.0       | 1.0            |
| H33258           | 4.1 ± 0.3        | 43.7 ± 1.3       | 2.4 ± 0.2       | 34.5 ± 1.4       | 0.6            |
| **Hoechst enhancement** |                  |                  |                |
| drug             | $K_d$ (μM)       | $\Delta F_{\text{max}}$ (× 10⁶) | $K_d$ (μM)       | $\Delta F_{\text{max}}$ (× 10⁶) | (fold change)$^1$ |
| H33342           | 11.8 ± 1.1       | 5.7 ± 0.2        | 8.6 ± 0.4       | 6.6 ± 0.1        | 0.7            |
| H33258           | 12.3 ± 1.5       | 3.4 ± 0.5        | 11.1 ± 0.7      | 3.9 ± 0.9        | 0.9            |

$^1$ fold-change = $(K_d)_{\text{Pgp-AzTMR}}/(K_d)_{\text{Pgp}}$
Figure 4.7  Binding affinity of Pgp for H33342 determined by Trp quenching and Hoechst fluorescence enhancement

Quenching of Trp fluorescence (Panel A) and enhancement of H33342 fluorescence (Panel B) for Pgp and Pgp-AzTMR on H33342 binding. Data are the average of 3 independent experiments ± SE. Data were corrected for buffer background, inner filter effect and dilution.

4.5 Binding of Clinically Important Drugs to Pgp and Pgp-AzTMR

Pgp has hundreds of substrates and the majority of them do not fit into the classical R- or H-site definition which was established for rhodamine and Hoechst compounds. Many of these Pgp substrates are drugs used clinically for the treatment of a variety of human medical conditions. The binding affinities of both Pgp and Pgp-AzTMR were determined for the anticancer agents cisplatin and daunorubicin, the gout medication colchicine, the calcium channel blocker verapamil, the calmodulin inhibitor trifluoperazine, the linear and cyclic peptides pepstatin A and cyclosporin A, respectively, and the lipid-like anti-cancer drugs ilmofosine and miltefosine (Table 4.3).

<table>
<thead>
<tr>
<th>Table 4.3</th>
<th>Binding affinity of Pgp and Pgp-AzTMR for clinically important drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pgp</td>
</tr>
<tr>
<td>drug</td>
<td>K_d (μM)</td>
</tr>
<tr>
<td>colchicine</td>
<td>214 ± 51</td>
</tr>
<tr>
<td>ilmofosine</td>
<td>44.9 ± 1.8</td>
</tr>
<tr>
<td>miltefosine</td>
<td>32.4 ± 1.2</td>
</tr>
<tr>
<td>pepstatin A</td>
<td>27.6 ± 1.2</td>
</tr>
<tr>
<td>cisplatin</td>
<td>16.8 ± 0.7</td>
</tr>
<tr>
<td>daunorubicin</td>
<td>13.6 ± 1.7</td>
</tr>
<tr>
<td>trifluoperazine</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>verapamil</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>cyclosporin A</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

\[ ^1 \text{fold-change} = \frac{(K_d)_{Pgp-AzTMR}}{(K_d)_{Pgp}} \]

The binding affinity of these compounds for Pgp and Pgp-AzTMR ranged from high (cyclosporin A, K_d = 0.8 μM) to low (colchicine, K_d = 214 μM). Several of the drugs...
(daunorubicin, colchicine, cisplatin, ilmofosine and miltefosine) bound to labelled Pgp with equal or higher affinity compared to unlabelled Pgp. The others (trifluoperazine, verapamil, pepstatin A and cyclosporin A) bound with equal or lower affinity to Pgp-AzTMR relative to unlabelled Pgp.

4.6 Pgp-AzTMR Drug Transport

In order to determine if crosslinking AzTMR to Pgp had any effect on its ability to transport drugs, a real-time fluorescence assay was used to measure the drug transport function of Pgp and Pgp-AzTMR. Both labelled and unlabelled Pgp were reconstituted into DMPC proteoliposomes at a lipid:protein ratio of approximately 18:1 (w/w). The reconstitution procedure yielded proteoliposomes which had moderate ATPase activity. The transport of TMR, H33342 and LDS-751 have all been measured previously using reconstituted Pgp, however, both TMR and LDS-751 had overlapping fluorescence properties with AzTMR, making their transport impossible to measure. A 5 μM concentration of H33342 was used in the transport experiments, as this concentration had previously been determined as optimal. The H33342 fluorescence ($\lambda_{ex}/\lambda_{em} = 354/450$ nm) in both Pgp and Pgp-AzTMR proteoliposomes decreased following the addition of ATP and an ATP regenerating system to initiate transport (Figure 4.8A). This occurs as the protein pumps the fluorescent drug out of the bilayer into the lumen of the liposome. Since H33342 fluorescence is high in a membrane environment and low in aqueous solution, this results in a decrease in fluorescence intensity. In order to show that the observed decrease in fluorescence was not due to dynamic repartitioning of the drug, a vanadate control was also carried out. When identical Pgp proteoliposome samples were trapped in a non-transporting state by the addition of the inhibitor vanadate, no decrease
Figure 4.8  H33342 transport by Pgp and Pgp-AzTMR proteoliposomes

Proteoliposomes containing Pgp (Panel A) or Pgp-AzTMR (Panel B) and DMPC were incubated at 26°C with 5 μM H33342 with (dashed lines) or without (solid lines) 93 μM ortho-vanadate. H33342 emission (450 nm) was monitored for 10 min following excitation at 355 nm, with ATP and an ATP regenerating system added at the time indicated by the arrow to initiate transport.
in H33342 fluorescence was observed (Figure 4.8B). The initial rate of drug transport (\% change in fluorescence intensity/s) was measured for H33342 transport by both Pgp and Pgp-AzTMR by taking the line slope for the first 20 s following the addition of ATP. The data were corrected for the vanadate control and protein concentration (Section 2.6.2), as the Pgp-containing liposomes had a slightly higher protein concentration than those of Pgp-AzTMR. The initial transport rates for unlabelled and labelled Pgp were $5.84 \times 10^5$ and $1.94 \times 10^5$ Au·s\(^{-1}\)·mg\(^{-1}\), respectively, representing a 3-fold faster rate of transport for Pgp compared to Pgp-AzTMR.

### 4.7 DISCUSSION

Previous work on the substrate binding pocket of Pgp revealed that drugs bind to Pgp within the cytoplasmic leaflet of the membrane. Fluorescence studies with the Pgp substrate, LDS-751, indicated that it binds with high affinity to a site that is relatively hydrophobic, with a polarity lower than that of chloroform (76). This is supported by the observation that many Pgp substrates are large non-polar/amphipathic molecules which are thought to interact with the protein via hydrophobic and van der Waals interactions. In the present study, a polarizability value was calculated for the AzTMR binding site from the measured Stokes shift that was similar to that of propanol. This is consistent with the relatively small blue shift seen for AzTMR compared to the larger blue shift observed for another Pgp substrate (76). Therefore, it appears that the binding location of AzTMR in Pgp is less polar than water, however, it is less hydrophobic than that of other drugs, likely reflecting the unique nature of each binding site.
The majority of drugs tested had a biphasic effect on Pgp ATPase activity, stimulating it up to a certain concentration and inhibiting it at higher concentrations. However, while TMR-E and TMR-M stimulated Pgp ATPase activity at low concentrations like the other rhodamines, they did not show inhibition at higher concentrations. Both AzTMR and LDS-751 were more inhibitory towards Pgp-AzTMR at low concentrations compared to unlabelled Pgp, but for the other drugs (both R-site and H-site) there was little change in the ATPase profiles of labelled and unlabelled Pgp. Thus, AzTMR crosslinking to Pgp does not appear to affect the way drug-induced conformational changes are transmitted to the NBDs.

It is generally accepted that Pgp can bind more than one substrate simultaneously. In this chapter, the binding of a second R-site, H-site, or other drug was quantified by estimating a $K_d$ value for binding of each drug to Pgp-AzTMR and comparing it with binding to unlabelled Pgp. The majority of rhodamines (TMR, AzTMR, R123, R6G, R101, and R110) bound to Pgp-AzTMR with lower affinity than Pgp, indicating that they likely bind close to the site where AzTMR is linked. Taken together, these data indicate that AzTMR is very likely covalently crosslinked to the R-site of Pgp. Unexpectedly, the structurally similar compounds, TMR-M and TMR-E, actually bound to Pgp-AzTMR with higher affinity than Pgp, suggesting they may not bind to the same site as the other rhodamines. The effect of these compounds on ATPase activity was also unusual, and it is possible that the methyl- and ethyl-ester groups of these rhodamines (Figure 4.1) result in them binding to a different sub-site. The binding to Pgp-AzTMR of LDS-751, which was previously identified as an R-site drug (130), was consistent with the majority of rhodamines, in that it bound with lower affinity to Pgp-AzTMR, which is in agreement
with earlier observations from our lab (54). However, unpublished transport data from our lab (Balpreet Vinepal, 2008, M.Sc. thesis, University of Guelph) indicated that LDS-751 competes for transport of both TMR and H33342, indicating that it likely binds to both the H-site and the R-site.

While there was a 3- to 4-fold difference in the $K_d$ values obtained through intrinsic Trp fluorescence quenching or enhancement of bound Hoechst fluorescence, the trends seen for binding to labelled and unlabelled Pgp were similar. For Trp quenching, H33342 and H33258 bound to Pgp-AzTMR with similar and higher affinity, respectively. When determined by ligand fluorescence enhancement, both Hoechst drugs had a higher affinity for Pgp-AzTMR. These findings suggest that binding of AzTMR does not affect the binding of the Hoechst molecules, which is expected, since they likely interact with the H-site.

The finding that a majority of R-site drugs bound to Pgp-AzTMR with lower affinity suggests steric interference with binding. Since both H-site drugs bind with higher affinity, covalent linkage of AzTMR to the R-site may induce slight conformational changes in Pgp that encourage binding of these compounds. In a previous study, R-site drugs were found to stimulate the transport of H-site drugs (and vice versa), while two R- or H-site drug inhibited each others transport, consistent with the binding data (127).

The binding affinities of Pgp and Pgp-AzTMR for several clinically important drugs were determined. Some of the compounds bound to Pgp-AzTMR with higher affinity, and some with lower affinity, indicating that the presence of crosslinked AzTMR affected the binding of most of them. Because of the range in size and structure of the drugs
tested, they may bind to different sub-sites in the Pgp binding pocket. Importantly, all of
the compounds were able to bind to labelled Pgp, proving that they are capable of binding
even in the presence of linked AzTMR.

Both Pgp and Pgp-AzTMR were successfully reconstituted into DMPC proteoliposomes which were able to transport H33342. The initial rate of transport measured for Pgp-AzTMR was 3-fold lower than that observed for Pgp, indicating that it clearly retains significant activity after the crosslinking process. The presence of cross-linked TMR could lead to partial steric blocking of the transport pathway for H33342. It is possible, but unlikely, as Pgp-AzTMR retained ATPase activity, that the decrease in drug transport was due to irreversible damage caused by crosslinking. In addition, as several batches of Pgp-AzTMR were combined and concentrated prior to reconstitution, it is possible that AzTMR labelling of one or more of the batches was incomplete, so that residual transport activity arises from unlabelled Pgp. Recently, Chiba and coworkers identified two pseudosymmetric solute transport pathways in Pgp by site-directed mutagenesis of residues lining the translocation pocket (148). Moreover, they showed that Pgp substrates selectively, but not exclusively, use one of these pathways over the other. It is possible that covalent linkage of AzTMR to Pgp blocks the R-selective pathway but does not affect the ability of the H-selective pathway to function normally. Perhaps the coupling of communication between the TMDs and NBDs is also altered as a result of having a permanent occupant in the drug binding site.
Chapter 5

BINDING OF DRUG MONOMERS AND DIMERS TO PGP/PGP-AZTMR AND EFFECTS ON ATPASE ACTIVITY
5.1 **INTRODUCTION**

Pgp affects the uptake, distribution and bioavailability of clinically administered drugs. A major contributor to MDR in human cancer, Pgp also prevents entry of therapeutic drugs into the brain, and has become a highly sought after drug target \((18;21)\). Pgp reversal agents, or modulators, block Pgp-mediated function by binding to the protein themselves, and/or overwhelming the transporter in a futile transport cycle. However, the lack of efficacy of third generation modulators in clinical trials has cast serious doubt on their usefulness in reversing MDR in cancer treatment \((21;26;149)\). Pgp modulators may still be useful in enhancing drug delivery to the brain, which is a major problem in treating diseases such as HIV, Parkinson’s disease, and epilepsy \((131)\).

A relatively new concept in the rational design of Pgp modulation has been the development of bivalent Pgp inhibitors \((150;151)\). These compounds were developed based on existing evidence that Pgp can simultaneously bind two substrates \((54;55)\). It was proposed that polyvalent modulators would bind more tightly to Pgp and thus be more effective at inhibiting its action \((150)\). Early work with dimers of the insecticide stipiamide provided some clues as to the size and structure of the Pgp binding pocket \((150)\). Dimers with different tether lengths of the antiprotozoal emetine, the antiretroviral abacavir, the antimalarial quinine and the antipsychotic quetiapine (Figure 5.1) have all been developed, and are in various stages of testing with Pgp \((132;152)\). Using several emetine dimers of different lengths, an optimum distance of 10 Å between the monomeric halves was needed to inhibit R123 transport with an IC\(_{50}\) of 2.9 μM, and only 5 nM was needed to inhibit \(^{125}\)I-iodoarylazidoprazosin (IAAP) labelling by 50% \((152)\).
Figure 5.1 Structures of monomers and dimers used in this study
Moreover, one of the emetine dimers was found to reverse the MDR phenotype of MCF-7/DX1 cells when co-administered with doxorubicin.

Subsequent versions of quinine and quetiapine drug dimers were specifically designed to have a tether with a built-in clearance mechanism, and contained reversible ester bonds or disulfide linkages that can be broken down in tissues. Quinine is a known Pgp modulator and the quinine methylene dimer was found to be a potent inhibitor of R123 efflux from cultured cancer cell lines, with an IC$_{50}$ of 1.7 μM. Furthermore, quinine methylene dimer inhibited the transport of several fluorescent and radiolabelled Pgp substrates and, like the emetine dimers, prevented $^{[125]}$I-IAAP labelling of Pgp. Similar studies with the antipsychotic drug quetiapine and several dimers with different tether lengths are currently under way.

A major problem in the drug treatment of medical conditions affecting the CNS is the penetration of these agents across the BBB. The eradication of HIV reservoirs in the brain is difficult, as many HIV protease inhibitors are Pgp substrates and are removed before they accumulate. A recent study, using reversible prodrug dimers of the antiretroviral agent abacavir, found that not only did these agents block their own efflux by Pgp, they were also converted to the monomeric (and therapeutic) form upon entering the cell. This strategy may be useful for other therapies which have limited CNS penetration due to Pgp.

The majority of experiments with these drug dimers were conducted with cultured cells, or crude membrane vesicles expressing Pgp, limiting the amount of information that could be obtained. Therefore, the study of these dimeric compounds in simpler systems is necessary to characterize their interactions with Pgp at the molecular level.
These drug dimers are useful probes to explore the size and binding capacity of the substrate-binding pocket of Pgp, especially when combined with the Pgp-AzTMR adduct. For example, if a large drug dimer is still able to bind to Pgp-AzTMR, this would indicate that the substrate binding pocket is large enough to accommodate three drug moieties. 

In the current study, monomers and dimers of abacavir, quinine and quetiapine (Figure 5.1) were tested for Pgp binding and their effect on Pgp ATPase activity. The direct measurement of the binding affinity of Pgp for these compounds is useful in evaluating their overall performance as potential modulators, and in the design of more effective agents. In addition, the use of these compounds combined with the Pgp-AzTMR adduct can provide some important information on the size of the substrate-binding pocket, and its ability to accommodate multiple drug units.

## 5.2 ABACAVIR MONOMER AND DIMERS

### 5.2.1 Binding affinity of Pgp and Pgp-AzTMR for abacavirs

The binding affinities of Pgp and Pgp-AzTMR for abacavir and two of its dimers, abacavir disulfide dimer and abacavir methylene dimer (Figure 5.2), were determined by measuring the intrinsic Trp quenching ($\lambda_{ex}/\lambda_{em} = 290/330$ nm) of the two proteins. Figure 5.2A shows that both dimers quenched the intrinsic Trp fluorescence of Pgp to a similar extent as the monomer, but at much lower concentrations. For Pgp, the estimated $K_d$ values for the disulfide and methylene dimers were much lower than the $K_d$ value for the monomer, while the maximum quenching for each ($\Delta F_{max}$) was similar at $\sim$20% (Table 5.1). Thus, abacavir disulfide and methylene dimers bind to Pgp with 6- to 8-fold higher affinity than the abacavir monomer, likely the result of an increased number of contacts with the protein side chains in the binding pocket, and more van der Waals interactions.
Figure 5.2  Quenching of Pgp and Pgp-AzTMR Trp fluorescence by abacavirs

Quenching of Trp fluorescence of Pgp (Panel A) and Pgp-AzTMR (Panel B) by abacavir monomer and dimers. Data are the average of 3 independent experiments ± SE. Data were corrected for buffer background, inner filter effect and dilution. Where not visible, error bars are contained within the symbols.
Table 5.1 Binding affinity of Pgp and Pgp-AzTMR for abacavirs

<table>
<thead>
<tr>
<th>compound</th>
<th>MW (kDa)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
<th>relative $K_d$ monomer/dimer(^1)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
<th>relative $K_d$ monomer/dimer(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>abacavir monomer</td>
<td>286</td>
<td>32.5 ± 6.5</td>
<td>23.7 ± 1.3</td>
<td></td>
<td>29.7 ± 5.3</td>
<td>18.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>disulfide dimer</td>
<td>775</td>
<td>5.30 ± 0.27</td>
<td>23.2 ± 0.4</td>
<td>6.1</td>
<td>4.79 ± 0.44</td>
<td>19.6 ± 0.5</td>
<td>6.2</td>
</tr>
<tr>
<td>methylene dimer</td>
<td>739</td>
<td>4.12 ± 0.48</td>
<td>21.5 ± 0.7</td>
<td>7.9</td>
<td>5.18 ± 1.46</td>
<td>19.7 ± 1.6</td>
<td>5.7</td>
</tr>
</tbody>
</table>

\(^1\) relative $K_d = (\text{monomer } K_d)_{\text{Pgp}}/(\text{dimer } K_d)_{\text{Pgp}}$

\(^2\) relative $K_d = (\text{monomer } K_d)_{\text{Pgp-AzTMR}}/(\text{dimer } K_d)_{\text{Pgp-AzTMR}}$
The effect of all three compounds on Pgp-AzTMR was similar to that of unlabelled protein; all quenched ~20% of the intrinsic Trp fluorescence, with the two dimers doing so at substantially lower concentrations (Figure 5.2B). The $K_d$ values for the abacavir dimers were decreased ~6-fold relative to the monomer (Table 5.1). These results indicate that the absolute and relative affinities of Pgp for the abacavirs are unaffected by AzTMR labelling. Moreover, the binding affinities of Pgp and Pgp-AzTMR were similar for the monomer and both dimers (Table 5.1). This suggests that both halves of the dimer are able to fit in the binding pocket in addition to the linked TMR group (Figure 5.3), otherwise the affinity would be equal to that of the monomer, and that three drug moieties can fit in the binding pocket of Pgp.

Figure 5.3 Drug dimer binding to Pgp-AzTMR

Upon binding to Pgp, the drug dimers could bind with both drug moieties inside the binding pocket (left), or one drug moiety inside and the other outside the pocket (right).
5.3 QUININE MONOMER AND DIMERS

5.3.1 Binding affinity of Pgp and Pgp-AzTMR for quinines

The binding affinity of Pgp and Pgp-AzTMR for quinine and its methylene and disulfide dimers (Figure 5.1) was determined by measuring the intrinsic Trp quenching ($\lambda_{ex}/\lambda_{em} = 290/330$ nm) of Pgp (Figure 5.4A). Quinine quenched Pgp Trp fluorescence by ~30%, while both dimers quenched Trp fluorescence by ~55%; the estimated $K_d$ values are shown in Table 5.2. These results indicate that the quinine methylene and disulfide dimers bind to Pgp with 20- and 27-fold greater affinity than quinine, indicating greatly increased interactions of the dimers inside the drug binding pocket, compared to the monomer.

For Pgp-AzTMR, quinine and both quinine dimers quenched Trp fluorescence by ~20% and 45%, respectively, and the binding affinity of Pgp-AzTMR for both dimers was much higher than that of quinine (Figure 5.4B). The $K_d$ values (Table 5.2) indicate that both dimers bind to Pgp-AzTMR with 11- to 12-fold higher affinity than the quinine monomer. Therefore, the entire quinine dimer can likely fit into the drug-binding pocket of Pgp (Figure 5.3), otherwise the affinity would be lower, similar to that of quinine.

5.3.2 Effect on ATPase activity

The effect of quinine and its two dimers on the ATPase activity of Pgp and Pgp-AzTMR was determined at various concentrations from 0.6-300 μM. Quinine stimulated Pgp ATPase activity by 10-20% up to a concentration of 200 μM, whereas both quinine methylene and disulfide dimers had similar effects on activity, inhibiting it at all concentrations to a maximum of 80% and 60%, respectively (Figure 5.5A). These results indicate that the second quinine molecule in the dimers must somehow shift the drug
Figure 5.4  Quenching of Pgp and Pgp-AzTMR Trp fluorescence by quinines

Quenching of Trp fluorescence of Pgp (Panel A) and Pgp-AzTMR (Panel B) by quinine monomer and dimers. Data are the average of 3 independent experiments ± SE. Data were corrected for buffer background, inner filter effect and dilution. Where not visible, error bars are contained within the symbols.
<table>
<thead>
<tr>
<th>compound</th>
<th>MW (kDa)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
<th>relative $K_d$ monomer/dimer$^1$</th>
<th>$K_d$ (μM)</th>
<th>$\Delta F_{\text{max}}$ (%)</th>
<th>relative $K_d$ monomer/dimer$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>quinine monomer</td>
<td>384</td>
<td>20.5 ± 1.5</td>
<td>35.2 ± 1.0</td>
<td></td>
<td>18.2 ± 4.7</td>
<td>19.9 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>methylene dimer</td>
<td>793</td>
<td>1.01 ± 0.04</td>
<td>56.8 ± 0.5</td>
<td>20</td>
<td>1.57 ± 0.27</td>
<td>48.2 ± 1.2</td>
<td>11</td>
</tr>
<tr>
<td>disulfide dimer</td>
<td>835</td>
<td>0.75 ± 0.03</td>
<td>52.3 ± 0.4</td>
<td>27</td>
<td>1.50 ± 0.23</td>
<td>46.5 ± 1.0</td>
<td>12</td>
</tr>
</tbody>
</table>

$^1$ relative $K_d = (\text{monomer } K_d)_{\text{Pgp}}/(\text{dimer } K_d)_{\text{Pgp}}$

$^2$ relative $K_d = (\text{monomer } K_d)_{\text{Pgp-AzTMR}}/(\text{dimer } K_d)_{\text{Pgp-AzTMR}}$
Figure 5.5  Effect of quinines on ATPase activity of Pgp and Pgp-AzTMR

The ATPase activity of Pgp (Panel A) and Pgp-AzTMR (Panel B) was measured in the presence of various concentrations of quinine monomer and dimers ranging from 0.6 μM to 300 μM. Each data point is the average of triplicate measurements ± SE. The DMSO control was used as the 100% activity control. Where not visible, error bars are contained within the symbols.
from stimulating to inhibiting the ATPase activity of Pgp, suggesting a fundamental difference in drug coupling between the binding pocket and NBDs for the monomer and dimers. The effect of all three molecules on the ATPase activity of Pgp-AzTMR was similar to that of Pgp (Figure 5.5B), suggesting that crosslinking AzTMR in the binding pocket of Pgp does not greatly affect the coupling between the drug binding site and the NBDs. Quinine caused little stimulation of ATPase activity for both Pgp and Pgp-AzTMR, and inhibition levels were low (< 20%) at the highest concentration tested. Both quinine dimers inhibited Pgp and Pgp-AzTMR activity to a much greater extent, up to 80% at high concentrations, and did not stimulate activity at low concentrations.

5.4 QUETIAPINE MONOMER AND DIMERS

5.4.1 Binding affinity of Pgp and Pgp-AzTMR for quetiapines

The binding affinities of Pgp and Pgp-AzTMR for the quetiapine monomer, its disulfide, methylene (C2-Me2) and carbonate dimers and eight diester dimers (C1 to C8) (Figure 5.1) were determined by measuring the quenching of intrinsic Trp fluorescence ($\lambda_{ex}/\lambda_{em} = 290/330$ nm). The quetiapine monomer quenched Pgp Trp fluorescence to ~60% (Figure 5.6A), whereas each of the dimers had $\Delta F_{\text{max}}$ values of ~38% (C2-Me2)-49% (C1 diester). Each one of the dimers saturably quenched Trp fluorescence at much lower concentrations than quetiapine. The $K_d$ values ranged from a high of 2.49 $\mu$M (C8 diester) to a low of 0.96 $\mu$M (C3 diester), indicating that the dimers bind to Pgp with 12-to 30-fold higher affinity than the quetiapine monomer (Table 5.3).

Quetiapine was found to quench the Trp fluorescence of both Pgp and Pgp-AzTMR to a similar extent (Figure 5.7A). Interestingly, none of the dimers quenched the Trp fluorescence of Pgp-AzTMR to the same extent as they did for Pgp, with $\Delta F_{\text{max}}$ ranging
Figure 5.6  Binding of quetiapine monomer and dimers to Pgp

The quenching of Trp fluorescence of Pgp by quetiapine and a representative dimer, C6 is shown in Panel A. The extracted $K_d$ ($\bullet$) and $\Delta F_{max}$ ($\triangle$) values for each quetiapine dimer (arranged by size) are shown in Panel B. Data are the average of 3 independent experiments $\pm$ SE. Data were corrected for buffer background, inner filter effect and dilution. Where not visible, error bars are contained within the symbols.
<table>
<thead>
<tr>
<th>quetiapine compound</th>
<th>MW (kDa)</th>
<th>Kd (μM)</th>
<th>ΔF_{\text{max}} (%) relative Kd&lt;sup&gt;1&lt;/sup&gt; monomer/dimer</th>
<th>Kd (μM)</th>
<th>ΔF_{\text{max}} (%) relative Kd&lt;sup&gt;2&lt;/sup&gt; monomer/dimer</th>
<th>relative Kd&lt;sup&gt;3&lt;/sup&gt; Pgp-AzTMR/Pgp</th>
</tr>
</thead>
<tbody>
<tr>
<td>monomer</td>
<td>384</td>
<td>29.2 ± 2.9</td>
<td>64.5 ± 2.7</td>
<td>27.0 ± 4.0</td>
<td>54.5 ± 3.9</td>
<td>0.9</td>
</tr>
<tr>
<td>carbonate</td>
<td>793</td>
<td>1.50 ± 0.14</td>
<td>48.1 ± 1.0</td>
<td>1.14 ± 0.16</td>
<td>34.4 ± 1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>C1 diester</td>
<td>835</td>
<td>1.66 ± 0.12</td>
<td>48.6 ± 0.9</td>
<td>1.24 ± 0.19</td>
<td>35.1 ± 1.3</td>
<td>22</td>
</tr>
<tr>
<td>C2 diester</td>
<td>849</td>
<td>1.34 ± 0.10</td>
<td>44.9 ± 0.8</td>
<td>1.30 ± 0.16</td>
<td>39.9 ± 1.3</td>
<td>21</td>
</tr>
<tr>
<td>C2-Me2</td>
<td>877</td>
<td>0.99 ± 0.10</td>
<td>37.8 ± 0.9</td>
<td>0.50 ± 0.14</td>
<td>23.9 ± 1.2</td>
<td>54</td>
</tr>
<tr>
<td>C3 diester</td>
<td>863</td>
<td>0.96 ± 0.07</td>
<td>41.4 ± 0.6</td>
<td>0.80 ± 0.12</td>
<td>32.8 ± 1.1</td>
<td>34</td>
</tr>
<tr>
<td>C4 diester</td>
<td>877</td>
<td>1.02 ± 0.07</td>
<td>41.0 ± 0.6</td>
<td>0.43 ± 0.14</td>
<td>20.5 ± 1.1</td>
<td>63</td>
</tr>
<tr>
<td>C5 diester</td>
<td>891</td>
<td>1.00 ± 0.09</td>
<td>38.4 ± 0.7</td>
<td>0.82 ± 0.18</td>
<td>20.4 ± 1.0</td>
<td>33</td>
</tr>
<tr>
<td>C6 diester</td>
<td>905</td>
<td>1.31 ± 0.05</td>
<td>40.4 ± 0.3</td>
<td>1.02 ± 0.08</td>
<td>29.6 ± 0.5</td>
<td>26</td>
</tr>
<tr>
<td>C7 diester</td>
<td>919</td>
<td>2.45 ± 0.19</td>
<td>45.4 ± 1.0</td>
<td>1.31 ± 0.13</td>
<td>21.8 ± 0.6</td>
<td>21</td>
</tr>
<tr>
<td>C8 diester</td>
<td>933</td>
<td>2.49 ± 0.21</td>
<td>46.2 ± 1.1</td>
<td>1.24 ± 0.16</td>
<td>27.4 ± 0.9</td>
<td>22</td>
</tr>
<tr>
<td>disulfide</td>
<td>969</td>
<td>2.16 ± 0.19</td>
<td>42.5 ± 0.9</td>
<td>1.16 ± 0.12</td>
<td>24.3 ± 0.8</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>1</sup> relative K_d = (monomer K_d)<sub>Pgp</sub>/(dimer K_d)<sub>Pgp</sub>
<sup>2</sup> relative K_d = (monomer K_d)<sub>Pgp-AzTMR</sub>/(dimer K_d)<sub>Pgp-AzTMR</sub>
<sup>3</sup> relative K_d = (K_d)<sub>Pgp-AzTMR</sub>/(K_d)<sub>Pgp</sub>
Figure 5.7  Binding of quetiapine monomer and dimers to Pgp-AzTMR

The quenching of Trp fluorescence of Pgp-AzTMR by quetiapine and a representative dimer, C6 is shown in Panel A. The extracted $K_d$ (●) and $\Delta F_{\text{max}}$ (△) values for each quetiapine dimer (arranged by size) are shown in Panel B. Data are the average of 3 independent experiments ± SE. Data were corrected for buffer background, inner filter effect and dilution. Where not visible, error bars are contained within the symbols.
from 20-40%. Each of the dimers had much lower $K_d$ values for binding to Pgp-AzTMR when compared to quetiapine monomer, ranging from 1.31 μM (C7 diester) to 0.43 μM (C4 diester), representing 21- to 63-fold higher binding affinities than the monomer. When the binding of the monomer and dimers to labelled and unlabelled Pgp were compared, most of the compounds had a higher affinity for Pgp-AzTMR (Table 5.3). The fold-change in binding varied between 0.5 and 1.0, indicating that the binding of the quetiapines to Pgp was only slightly affected by the presence of AzTMR in the binding pocket (Table 5.3) and that three drug moieties can simultaneously fit into the binding pocket. When $K_d$ and $\Delta F_{\text{max}}$ were plotted against the size of the dimers, C2-Me2, C3, C4 and C5 were found to bind to Pgp with the highest affinity, suggesting that their tether lengths are optimal for fitting into the native Pgp drug-binding pocket (Figure 5.6B).

5.4.2 Effect on ATPase activity

The effect of the quetiapine monomer and dimers on Pgp and Pgp-AzTMR ATPase activity was measured over concentrations from 0.6-300 μM. From these curves, IC$_{50}$ values were estimated by determining the concentration at which 50% inhibition occurred (Table 5.4). Quetiapine showed a biphasic effect on Pgp ATPase activity, stimulating it 20-30% at concentrations up to 6 μM and inhibiting activity at higher concentrations, with an IC$_{50}$ of 15 μM. Using the calculated $K_d$ values, the fractional or site occupancy was calculated for each compound for the range of quetiapine concentrations used in the ATPase assay. At 2 μM, quetiapine was found to stimulate ATPase activity by 30% with only 6% of the drug binding sites occupied (Figure 5.9A). In general, the dimers from the shortest tether of C1 up to C5 stimulated Pgp ATPase activity at low concentrations and inhibited activity at concentrations above 1 μM (Figure 5.8A). The dimers with longer
tether lengths, C6 to C8, did not stimulate ATPase activity, but rather inhibited activity over the entire concentration range (Figure 5.8B). Although C2-Me2 has the same tether length as C2 and the same mass as C4, it did not show stimulation at low concentrations, but rather inhibited ATPase activity at all concentrations in a similar pattern to the quetiapines with longer tethers (C6 through C8). It is evident that additional methyl substituents affect the positioning of the tether within the binding pocket and, consequently, coupled ATPase activity. Quetiapine carbonate behaved much like the short tether-length diesters, stimulating ~10% at lower concentrations and inhibiting activity at higher values, whereas quetiapine disulfide behaved like the longer tethers, showing no stimulation at low concentrations. The IC50 values of the quetiapine dimer series decreased with increasing tether length, and increased at the longest tether C8 (Table 5.4).

The effect of quetiapine monomer and dimers on the ATPase activity of Pgp-AzTMR was also determined. Quetiapine monomer did not stimulate the activity of labelled Pgp at low concentrations, however, the overall shape of the ATPase profile is quite similar, and above 10 μM, quetiapine inhibited Pgp-AzTMR activity (Figure 5.9A). In addition, the IC50 value determined for quetiapine was much higher than those for each of the dimers, consistent with what was seen for Pgp (Table 5.4). Dimers with short tethers up to C3 stimulated Pgp-AzTMR ATPase activity at low concentrations and inhibited it at high concentrations (Figure 5.8A), while C4 and larger dimers only inhibited activity at all concentrations (Figure 5.8B). The carbonate dimer behaved like the short tethers and the disulfide behaved like the long tethers, similar to what was observed with Pgp. The only noticeable difference between the two data sets was that all
Figure 5.8 ATPase activity profiles of quetiapine C2 and C7 dimers with Pgp and Pgp-AzTMR.

Pgp and Pgp-AzTMR ATPase activity with increasing concentrations of C2 diester (Panel A) and C7 diester (Panel B) dimer and site occupancy (dashed line) calculated from the $K_d$ value are plotted on each graph. Each data point is the average of triplicate measurements ± SE. The DMSO control was used as the 100% activity control. Where not visible, error bars are contained within the symbols.
Figure 5.9  ATPase activity of quetiapine and IC_{50} of quetiapine dimers

The ATPase activity of Pgp and Pgp-AzTMR with increasing concentrations of quetiapine is shown in Panel A with site occupancy (dashed line) calculated from the K_{d} value. The IC_{50} values of the Pgp and Pgp-AzTMR are shown for each of the quetiapine dimers, arranged by size, in Panel B. The IC_{50} of Pgp for C2-Me2 (\(\times\)) and Pgp-AzTMR for C2-Me2 (\(\triangle\)) are indicated. The ATPase data are the average of triplicate measurements ± SE. The DMSO control was used as the 100% activity control. Where not visible, error bars are contained within the symbols.
the compounds had higher IC$_{50}$ values for labelled Pgp compared to unlabelled Pgp (Figure 5.9B). Altogether, the pattern of ATPase modulation by quetiapine and its dimers was very similar for Pgp and Pgp-AzTMR, indicating that crosslinking has little effect on drug coupling to the NBDs.

| Table 5.4 | IC$_{50}$ values for inhibition of Pgp and Pgp-AzTMR ATPase activity by quetiapines |
|-----------|----------------------------------|-----------------|-----------------|
| Compound  | MW (kDa)                         | Pgp IC$_{50}$ (μM) | Pgp-AzTMR IC$_{50}$ (μM) |
| Monomer   | 384                              | 14.9             | 52.1             |
| Carbonate | 793                              | 2.12             | 3.83             |
| C1 diester| 835                              | 2.06             | 2.03             |
| C2 diester| 849                              | 1.46             | 1.91             |
| C2-Me2    | 877                              | 0.50             | 1.30             |
| C3 diester| 863                              | 1.04             | 1.82             |
| C4 diester| 877                              | 0.56             | 1.56             |
| C5 diester| 891                              | 0.61             | 1.21             |
| C6 diester| 905                              | 0.56             | 1.32             |
| C7 diester| 919                              | 0.68             | 3.17             |
| C8 diester| 933                              | 1.52             | 3.32             |
| Disulfide | 969                              | 1.34             | 3.88             |

5.5 DISCUSSION

Pgp has long been considered an attractive drug target because of its demonstrated role in MDR. However, its polyspecificity has made it difficult to design MDR reversal agents. Many compounds that are known to modulate Pgp activity are simply not effective at clinically reversing MDR in patients, although many are very effective in vitro. The concept of using bivalent homodimers separated by spacers of a defined length to reverse Pgp-induced MDR is a novel approach based on the premise that two linked modulator molecules would amplify the inhibitory effects of a single molecule. Even if
these compounds proved to be unsuitable for clinical use, they might be useful in providing information on the size and nature of the Pgp drug-binding pocket. Several bivalent drug dimers have shown promising results in vitro by their ability to inhibit drug binding to Pgp and reduce Pgp-mediated drug efflux from cells (150). For example, a bivalent emetine probe was able to reverse the MDR phenotype of cells when administered with the cytotoxic agent daunorubicin (152). However, early versions of polyvalent modulators were thought to be too cytotoxic because they accumulated in cells and were not efficiently cleared. As a result, homodimers linked together with reversible ester bonds were developed. They were more quickly hydrolyzed by cytoplasmic esterases, while maintaining their ability to reverse MDR in vitro (132).

In the current study, the binding affinity and effects on ATPase activity of bivalent abacavir, quinine and quetiapine homodimers were measured with unlabelled Pgp, and Ppg labelled with the R-site drug, AzTMR. Interestingly, the abacavir, quinine, and quetiapine dimers bound to Pgp and Pgp-AzTMR with 6- to 8-fold, 11- to 27-fold, and 12- to 63-fold higher affinity, respectively. The fact that the drug dimers bind more tightly to Pgp than the monomer, which confirms the early hypotheses that dimers could be more potent inhibitors of Pgp, is likely the result of increased contacts and interactions inside the binding pocket. Furthermore, since the binding affinity of Pgp for modulator dimers is much higher compared to monomers, this is a useful strategy to improve their potency, as lower concentrations of modulators would be needed to reverse MDR if they are dimerized. The results also indicate that none of the dimers are prevented from binding to Pgp by the presence of linked AzTMR, and confirm that it may be possible for three drugs to simultaneously bind to Pgp. Indeed, that the binding pocket of Pgp may be
sufficiently large to accommodate at least two molecules of substrate is suggested by the recent X-ray crystal structure (40). The finding that the $K_d$ of each of the drug dimers was similar for labelled and unlabelled Pgp suggests that these compounds likely bind to a different sub-site within the binding pocket, which does not overlap with the R-site.

The effects of the drug monomers and dimers on the ATPase activity of Pgp and Pgp-AzTMR were complex and not easily explained. Both quinine dimers were much more potent inhibitors of ATPase activity than the monomer. However, there remains no satisfactory explanation for the observed ATPase profiles in the greater context of Pgp function, and the ability to inhibit ATPase activity alone does not necessarily make a compound a good Pgp modulator.

In the case of quetiapine, a large series of cross-linked dimers with different tether lengths was available, and provided some interesting information on the Pgp drug binding pocket. The extent to which the dimers quenched Pgp-AzTMR Trp fluorescence was lower than for Pgp, indicating that they may bind to a somewhat different site in labelled Pgp. Moreover, while quetiapine binds to labelled and unlabelled Pgp with similar affinity, each of the dimers had a higher affinity for Pgp-AzTMR, implying that this new site is more favourable for dimer binding. Of particular interest is the Pgp binding affinity for dimers with different tether lengths. The affinity increased with increasing tether length up to the C3 diester dimer and was equally strong for C3, C4, and C5. However, for C6 diester dimer and longer, the affinity decreased, suggesting that the optimum tether length for binding of bivalent quetiapines to Pgp is between C3 and C5. This trend was unchanged for binding to Pgp-AzTMR, with C3 to C5 diester having the highest binding affinities. These results are supported by earlier findings that the greatest
inhibition of $[^{125}\text{I}]-\text{IAAP}$ labelling of Pgp and R123 transport by Pgp, were achieved by stipiamide and emetine dimers with optimal tether lengths ($150;152$). Interestingly, the different chemical linkages in the carbonate, disulfide and C2-Me2 dimers were largely irrelevant to binding, as their affinities followed the trend based on tether length.

The biphasic pattern of ATPase stimulation and inhibition observed for monomeric quetiapine was also seen for short tether-length quetiapine dimers (up to C5), while the dimers with longer tether lengths (C6 and above) only inhibited. Their effects on verapamil-stimulated Pgp ATPase activity in crude membranes were comparable (C.A. Hrycyna, unpublished work). These results are also similar to the effects of stipiamide dimers on verapamil-induced ATPase activity, with activity stimulated by dimers with short tethers up to a certain threshold and inhibited by longer tethers ($150$). The only difference noted for Pgp-AzTMR was that the IC$_{50}$ values were noticeably higher compared to Pgp, indicating that the dimers are not as potent inhibitors of labelled Pgp ATPase activity.

Taken together, the results of these experiments with three monomer/dimer pairs with purified Pgp provide some useful information on the size of the Pgp binding pocket, and the relationship between the sub-sites where they bind and the R-site where TMR is linked.
Chapter 6

SUMMARY AND CONCLUSIONS
6.1 SUMMARY AND CONCLUSIONS

In this project, the fluorescent azido derivative of the Pgp substrate, TMR, was covalently crosslinked to the drug binding pocket by UV activation. Although previous studies crosslinked drugs to Pgp, they looked largely at the changes in ATPase activity and did not measure the direct binding of drugs to the crosslinked protein (91). Moreover, by using specific labelling at inserted Cys residues, rather than crosslinking to native residues present in the binding pocket, the physiological relevance of the results come into question. In this study, the small, highly reactive azido group was used for crosslinking to both minimize chemical changes to the substrate molecule, and attempt to link it to its natural site within the Pgp binding pocket. The ATPase activity of the Pgp-AzTMR adduct decreased relative to Pgp, however, this decrease was similar to that observed for Pgp with reversibly bound AzTMR, and could have resulted from a shift from the basal to the drug-coupled ATPase cycle (124). Reaction parameters were optimized to obtain covalently crosslinked Pgp with good functionality and a molar labelling stoichiometry of ~1. Other than its effect on ATPase activity, crosslinking of AzTMR to the drug binding pocket resulted in a Pgp-AzTMR adduct that displayed similar biochemical properties to native Pgp. It also retained the ability to transport H33342, albeit not to the same extent as native Pgp.

The crosslinking of AzTMR to Pgp resulted in a small blue shift in intrinsic Trp fluorescence, and the local environment of the binding site was estimated to have a polarity similar to that of propanol. Interestingly, this binding region is more polar than that for the substrate LDS-751, which was previously found to have a polarity lower than
chloroform (76). These results demonstrate that in order to achieve its polyspecificity, Pgp contains several distinct binding regions for different substrates.

Trp quenching of the Pgp-AzTMR adduct allowed the measurement of $K_d$ for binding of a second drug, without the complicating effects of multiple reversible drug equilibria that were encountered in previous studies (54). The binding affinity of labelled and unlabelled Pgp for a second substrate (rhodamine, Hoechst dye, or another clinically used drug) was determined. The majority of rhodamines tested had a higher affinity for Pgp than Pgp-AzTMR, suggesting that AzTMR may be linked close to their binding site, resulting in some steric interference. The binding affinity for two Hoechst compounds, measured by either intrinsic Trp fluorescence quenching or enhanced ligand fluorescence, was either unchanged or higher for Pgp-AzTMR. These results indicate that binding of H-site drugs to Pgp is not affected by a crosslinked molecule of AzTMR, and may even be made more favourable. The ATPase profiles of labelled and unlabelled Pgp were relatively similar for the majority of R- and H-site drugs, suggesting that crosslinking AzTMR to the R-site did not affect communication between the drug-binding pocket and the NBDs. The effect of AzTMR labelling on the binding affinities of several clinically important drugs to Pgp was variable, with some drugs binding more tightly to Pgp and others more tightly to Pgp-AzTMR. Therefore, the simple R- and H-site proposal for the Pgp binding site is likely more complicated than previously thought, involving broader R- and H-preferring regions which can bind a variety of molecules at overlapping sites. All of these results do, however, provide direct confirmation of simultaneous binding of two drug molecules to the Pgp substrate binding pocket (54;55).
Three sets of Pgp modulators and their potentially clinically useful dimers (quinine, quetiapine, and abacavir series) were used with AzTMR-labelled and unlabelled Pgp to obtain information on the size and capacity of the drug-binding pocket. In all cases, the dimers bound to Pgp with substantially higher affinity than the monomers, with between 6- and 30-fold lower $K_d$ values. This higher binding affinity likely results from increased contacts and interactions between the dimers and the binding pocket. Therefore, using drug dimers represents a useful strategy to enhance the Pgp binding affinity of these compounds. Quinine dimers and longer tether-length quetiapine dimers inhibited Pgp ATPase activity, unlike the monomers, which generally stimulated activity at low concentrations and inhibited it at high concentrations, producing a biphasic profile. An optimum tether length for quetiapine was found, between the C3 and C5 diester, for which the Pgp binding affinity was highest, and inhibition of ATPase activity the most potent. All of these results are consistent with earlier ATPase activity measurements made with monomer/dimer compounds using Pgp in crude membranes (132;150;152), and demonstrate the potential of these drugs as clinical Pgp-reversal agents.

The binding affinity of Pgp-AzTMR for the drug dimers was comparable to that noted for unlabelled Pgp, suggesting that the entire drug dimer is able to fit into the binding pocket together with the AzTMR group. These results suggest that AzTMR crosslinking does not significantly impact the dimers’ ability to interact with Pgp, and in fact, provides evidence that it is possible for three drug moieties to bind to the Pgp binding pocket simultaneously. Chemotherapy treatment is often carried out with drug cocktails containing several Pgp substrates. These results suggest that they would affect each others binding and transport, although the details cannot be predicted presently.


6.2 SUGGESTIONS FOR FUTURE WORK

It would be extremely useful to know the physical location of the AzTMR binding site in Pgp. To this end, mass spectrometry was employed as part of my studies to find the labelled peptide fragment from purified Pgp-AzTMR digested with trypsin and chymotrypsin. While ~30% sequence coverage was obtained, the fragments were largely from the extracellular and cytoplasmic domains; the coverage from the TMDs was very poor. Some groups have reported better sequence coverage for Pgp digested with chymotrypsin, especially in the TMDs, but these studies were completed on Pgp reconstituted into proteoliposomes and the details of their digestion protocol were not published (153). Therefore, while technically difficult, it should be possible to identify the labelling site of AzTMR in a carefully designed mass spectrometric analysis.

In this study, the binding affinities of a number of R- and H-site drugs were determined for Pgp with and without a crosslinked AzTMR label. H33258 can bind to Pgp-AzTMR and its fluorescence emission spectrum overlaps the excitation spectrum of AzTMR, with a calculated Förster distance of 53 Å for the pair of fluorophores. Therefore, FRET may occur between covalently-linked AzTMR and bound H33258, thus allowing the estimation of the distance between the two probes (77). This information could provide substantial insight into the relative location of the R- and H-sites within the binding pocket of Pgp.

In future studies, it might also be possible to covalently crosslink two drug molecules to Pgp in a sequential manner. Crosslinking of ethidium monoazide has been carried out in our lab (Richard Mather, 2008, M.Sc. thesis, University of Guelph), however, it is not strictly an R- or H-site drug. Therefore, it would be useful to synthesize
an azido derivative of either H33342 or H33258 to see if it is possible to covalently crosslink a second drug molecule to Pgp. This could give complementary information on the binding sub-site for H-site drugs and would also allow the evaluation of binding of a third drug to Pgp, using similar approaches to those in this thesis.

The bivalent versions of Pgp modulators show promise in their ability to act as MDR-reversal agents by inhibiting drug binding to Pgp at much lower concentrations than the monomers (150). These compounds have also been shown to inhibit R123 efflux from cells, and it should be possible to measure their effect on H33342 transport using Pgp reconstituted into DMPC proteoliposomes. Moreover, measurement of the binding affinity of these compounds to Pgp covalently cross-linked to both AzTMR and an H-site drug could demonstrate whether four drugs can bind to Pgp simultaneously, and provide additional information on the maximum size of the binding pocket.

The interaction of nucleotides with Pgp and Pgp-AzTMR could provide valuable information on the coupling process between drug binding and ATP hydrolysis. In the ATP-switch model (123), drug binding is proposed to induce binding of ATP, which it is assumed cannot bind tightly in the absence of drug. However, the partition model (124), and data from our lab (32), suggest that drugs and ATP can bind independently of each other. By measuring Trp quenching of Pgp, the binding affinity of nucleotides (ATP and ADP) can be determined for Pgp and Pgp-AzTMR. Whether or not there is a significant difference in the binding affinity of nucleotide for both states of Pgp, this should provide some idea on how the presence of drug in the binding pocket affects nucleotide binding.
REFERENCES


77. Qu, Q. and Sharom, F. J. (2002) Proximity of bound Hoechst 33342 to the ATPase catalytic sites places the drug binding site of P-glycoprotein within the cytoplasmic membrane leaflet, Biochemistry 41, 4744-4752.


95. Qu, Q. and Sharom, F. J. (2001) FRET analysis indicates that the two ATPase active sites of the P-glycoprotein multidrug transporter are closely associated, *Biochemistry* 40, 1413-1422.


125


145. Pierce Applications Handbook and Catalog, (2005), Rockford, IL.


