Drug-Drug Interactions in The Binding Pocket of The
P-Glycoprotein Multidrug Transporter

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ABSTRACT

DRUG-DRUG INTERACTIONS IN THE BINDING POCKET OF THE
P-GLYCOPROTEIN MULTIDRUG TRANSPORTER

Danoo Vitsupakorn
University of Guelph, 2014
Advisor: Professor F.J. Sharom

The ABC multidrug transporter P-glycoprotein (Pgp, ABCB1) can transport structurally diverse substrates from the lipid bilayer. Pgp binds its substrates inside a large pocket with multiple sub-sites. In the present work, a thiol-reactive agent, (2-pyridyl)dithiobimane, was covalently linked to 5 of the 7 Cys residues in hamster Pgp, with substantial ATPase activity retained. Three of the labelled Cys residues are located in the transmembrane domain, close to the substrate-binding pocket. Binding affinity determination using Trp and bimane fluorescence for tetramethylrosamine (R-site), Hoechst 33342 (H-site) and 6-(dimethylamino)-2-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl perchlorate (both sites), showed similar $K_d$ values for native Pgp and Pgp-bimane adducts, suggesting that the binding pocket can accommodate more than one substrate simultaneously. Reconstituted Pgp-bimane proteoliposomes showed the ability to transport all three substrates across the membrane, although with lower $V_{max}$ values compared to native Pgp. Thus, the Pgp-bimane adduct can transport substrates when more than one substrate occupies the binding pocket.
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenosine-5’-(β,γ-imido)triphosphate</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</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>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DOC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>DTE</td>
<td>Dithioerythritol</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>H33342</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>LDS-751</td>
<td>6-(dimethylamino)-2-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl perchlorate</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MIANS</td>
<td>2-(4’-maleimidylanilino)naphthalene-6-sulfonic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance-associated protein</td>
</tr>
<tr>
<td>NATA</td>
<td>N-acetyltryptophan amide</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDT-bimane</td>
<td>(2-pyridyl)dithiobimane</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>SDFL</td>
<td>Site-directed fluorescence labelling</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMEA</td>
<td>Tris-(2-maleimidoethyl)amine</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrosamine</td>
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Chapter 1

Introduction
1. Introduction

1.1 P-glycoprotein

1.1.1 The ABC superfamily

ATP-Binding Cassette (ABC) proteins are integral membrane proteins that make up one of the largest families of membrane transport proteins. They are found in the membranes of Archaea, Eubacteria and Eukarya, where many of them translocate diverse substrates across the membrane (1,2). Genome sequencing has identified ABC proteins encoded in many species. There are 80 ABC transporter genes in *Escherichia coli* (3) and ~48 genes in humans (4). In general, ABC transporters consist of four core domains, two transmembrane domains (TMDs), which are embedded in the lipid bilayer membrane to provide the translocation pathway, and two nucleotide-binding domains (NBDs), which are located in the cytoplasm (Figure 1.1). There are many possible combinations of these four domains (5). In bacteria, they are often found to be expressed separately, as either two or four polypeptides. In contrast, in eukaryotes, ABC proteins are often expressed as a single polypeptide with an internal duplication. These ABC proteins share a highly conserved set of motifs in the NBDs, which bind and hydrolyze ATP to provide the driving force for translocation (6-8).

P-glycoprotein (Pgp/ABCB1/MDR1) is the mammalian ABC transporter that is the best known with respect to its structure, mechanism and clinical relevance. It was first discovered in cultured cell lines as a protein associated
Figure 1.1  Topology diagram showing the arrangement of the TMDs and NBDs of hamster Pgp in the plasma membrane

The 12 TM helices are embedded in the membrane. The 11 Trp residues are shown. Six residues are in the N-terminal half (three residues are in the TM helices and the rest in the intra/extracellular loops) and five residues are in the C-terminal half (one residue is in an extracellular loop and the rest are in the intracellular loops and the NBD) (9). Two Cys residues are in the N-terminal half, while the remaining 5 residues are in the C-terminal half. The two Cys residues that are the target of MIANS labelling are indicated as red circles in both NBDs. The Cys residues in the TMDs are shown as green circles. The other Cys residues are indicated as yellow circles.
with multidrug resistance (MDR) (10). Several classes of chemotherapeutic agents were involved in the development of this resistance; Pgp plays an important role in transporting these drugs out of cells, thus abolishing their cytotoxic effects (11). The substrate specificity of Pgp is very broad, but the principles by which its substrates are defined are not fully understood.

1.1.2 The physiological role of Pgp

Various tissues of humans and rodents were found to express Pgp, including the small and large intestine, adrenal gland, liver, kidney, placenta and capillary endothelial cells of the testis and brain (12,13). Most of them have a low level expression of Pgp except for the apical surface of epithelial cells of the large and small intestine, liver bile ductules and kidney proximal tubules, which have a much higher expression level. It is clear that the primary physiological role of Pgp in the blood-brain barrier and placenta is to prevent toxic agents from permeating into sensitive tissues (14,15). Also, Pgp located in the intestine helps limit the absorption and oral bioavailability of substrates. The inhibition of Pgp function can cause clinically significant drug interactions, and can also increase the penetration and the accumulation of drugs in the brain. A study on transgenic knockout mice lacking Pgp supports the idea of Pgp’s physiological role (16). Mice that lack Pgp are still normal and fertile until challenged with Pgp substrates. In this situation, the Pgp substrate accumulation in brain is higher than usual compared to wild-type mice, resulting in neurotoxicity. For example, sensitivity to ivermectin, a pesticide commonly used in mouse colonies, is 100-fold higher in Pgp-knockout mice than in wild-type mice.
1.1.3 Multidrug resistance

MDR is the phenomenon by which cancer cells develop resistance to anti-cancer drugs with unrelated structures and mechanisms of action. For example, cancer patients may respond well for the first series of chemotherapy treatments. But when the cancer recurs, it is found to be resistant, leading to patient death (17). MDR was first noted in cultured cell lines that were selected for growth in a high concentration of various drugs. Expression of higher levels of several ABC transporters was found, corresponding to the appearance of the MDR phenotype in cell lines. The first ABC transporter, Pgp, was discovered in Chinese hamster ovary (CHO) cell lines. The cell lines displayed resistance not only to colchicine, but also to a variety of other structurally unrelated compounds (10,18). A decade later the multidrug resistance-associated protein 1 (MRP1, ABCC1) was discovered in a human small-cell lung cancer cell line (H69) (19). Detection of lower drug accumulation and resistance to a broad range of anticancer agents was found in H69 cell lines without increased expression of Pgp (20). A third ABC transporter linked to MDR, named ABCG2 (also known as breast cancer resistance protein, BCRP, or mitoxantrone resistance protein, MXR), was found more recently (21). Clinically, expression of Pgp in humans has been linked to poor response to chemotherapy treatment, and poor patient survival. However, the paradigm of MDR has changed after the discovery of additional Pgp-independent mechanisms of drug resistance, since many other ABC transporters may also contribute. It has been suggested that at least 12 ABC transporters
from four ABC subfamilies confer drug resistance to cells maintained in tissue culture (22,23).

1.2 Structure and topology of Pgp

1.2.1 Pgp topology and sequence

Hamster, mouse, and human Pgp are expressed as a single polypeptide, which is organized into two homologous halves. Each half contains one TMD, which comprises six α-helices embedded in the lipid bilayer membrane to provide the translocation pathway, and one NBD, located on the cytoplasmic side of the membrane. The NBDs generate energy for efflux transport by hydrolyzing ATP. Each NBD includes three important conserved regions; the Walker A motif, the Walker B motif, and the ABC signature motif, which together bind and hydrolyze ATP. The percent identity of hamster (ID: AAA37004.1) or mouse (ID: AAA59575.1) Pgp compared to human (ID: AAA59575.1) is 87%, suggesting that hamster and mouse Pgp can be a good model for studying human Pgp. A topology model of Pgp is shown in Figure 1.1. Epitope insertion in mouse Pgp, and Cys scanning mutagenesis in Cys-less human Pgp, were carried out to collect biochemical data and to confirm the topology (24,25). The Pgp amino acid sequence from BLAST showed that it has 7 Cys residues, two in the N-terminal half and five in the C-terminal half. A Cys residue in the Walker A motif of each NBD has been used as a selective targeting site for Cys-specific labeling reagents (Figure 1.1) (26,27).
1.2.2 Crystal structure of Pgp

In 2009, three medium resolution (3.8-4.4 Å) X-ray crystal structures of mouse Pgp were determined (Figure 1.2) (28). To date, this is the only 3D structure that shows Pgp co-crystallized with a substrate inside the binding pocket. All of the structures (Figures 1.2A, B and C) appeared without nucleotide in the NBDs. Two of the crystal structures are in the presence of substrates, QZ59-RRR and QZ59-SSS, bound in the drug-binding pocket (Figures 1.2B and C). Both QZ59-RRR and QZ59-SSS are cyclic peptides, which are stereoisomers of each other. TMD α-helices from each half form the drug-binding pocket, and the protein is observed to be in an inward-facing conformation. The internal cavity of the large drug-binding pocket was estimated to be about 6000 Å³, with a 30 Å separation of the two NBDs. Interestingly, the drug binding pocket is able to accommodate two molecules of QZ59-SSS at the same time with a different location and orientation. Moreover, the substrate QZ59-RRR is bound in a middle site (Figure 1.2F) between the two binding sites of QZ59-SSS, the upper and lower sites (Figure 1.2G), in the drug-binding pocket. The crystal structure of mouse Pgp showed three Cys residues located in the TMDs, one in transmembrane helix (TM) 2, one in TM7, and one in TM11 (Figures 1.2D and E), and the rest are in the NBDs and intracellular loops.

A high-resolution crystal structure (3.4 Å) of *C. elegans* Pgp was recently published (29). The crystal structure, which shows an inward-facing conformation without nucleotide in the NBDs, resembles the structure of mouse Pgp.
Figure 1.2 X-ray crystal structures of mouse Pgp

The three dimensional structure of mouse Pgp without ATP and substrate (A) (PDB: 3G5U) is shown with the N-terminal half in orange (TMD1) and dark blue (NBD1), and the C-terminal half in cyan (TMD2) and yellow (NBD2). (B) and (C) show Pgp without ATP bound to one molecule of QZ59-RRR (PDB: 3G60) and two molecules of QZ59-SSS (PDB: 3G61), respectively. The three Cys residues (purple) in the drug-binding pocket of mouse Pgp are shown in top-view (D) and side-view (E). The different locations of two QZ59-SSS molecules are shown in the upper (blue) and lower (red) sites (G), while one molecule of QZ59-RRR (green) is located in between them (F).
Sequence assignment of individual TMs showed agreement for six helices (TM1, TM2, TM6, TM7, TM8 and TM11), while TM9, TM10 and TM12 were shown in different conformations. Comparable helices, including TM3, TM4, and TM5 showed important differences due to register shifts in model building. The TM cavity, which formed the drug transport pathway, was located from the cytoplasmic surface to the membrane inner leaflet, suggesting that drug could gain access to the transport pathway from both the aqueous and lipid phases.

1.3 Interaction of Pgp with drugs

1.3.1 Pgp substrates and modulators

There are two major groups of compounds that interact with Pgp, called substrates and modulators (also known as inhibitors or chemosensitizers). Pgp produces cellular resistance to substrates, by pumping them out of the cell, so all substrates are transported by the protein. In contrast, modulators reverse MDR, by interfering with the ability of Pgp to transport substrates (30,31). Both of them, in general, are structurally diverse, amphipathic and hydrophobic, and often contain aromatic rings with positively charged N atoms. Lists of some substrates and modulators are shown in Tables 1.1 and 1.2. In the case of substrates, drug transport has been measured directly for only a few of them. Most substrates have been identified using Pgp-overexpressing cell lines, based on their resistance and cytotoxicity effects. The other group, modulators, also interact with Pgp and inhibit its transport function, but the molecular mechanism of inhibition is still not clear. There are several ways that the modulators may act on
Pgp. For example, some modulators are targeted to the substrate binding-pocket in the TMDs, so they compete with other substrates to inhibit their transport by Pgp. Some of these modulators may be themselves transported by Pgp, for example, cyclosporin A (32). On the other hand, LY335979 (zosuquidar) can bind tightly to the binding-pocket but is not transported by Pgp (33). The substrate binding pocket in the TMDs is not the only target for modulators; a few of them can bind to the NBDs located in the cytosol and modulate ATP hydrolysis. For example, progesterone binds recombinant NBD1 (34) and flavonoids interact with recombinant NBD2 (35).

**Table 1.1 Pgp substrates**

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
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<tbody>
<tr>
<td>Anti-cancer drugs</td>
<td>Anthracyclines: doxorubicin, daunorubicin</td>
</tr>
<tr>
<td></td>
<td>Taxanes: paclitaxel, docetaxel</td>
</tr>
<tr>
<td></td>
<td>Vinca alkaloids: vinblastine, vincristine</td>
</tr>
<tr>
<td>Natural products</td>
<td>Flavonoids</td>
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<td></td>
<td>Curcuminoids</td>
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<tr>
<td></td>
<td>Colchicine</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>Fluorescent dyes</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td></td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td></td>
<td>Calcein AM (calcein acetoxymethylester)</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Ca(^{2+}) channel blockers</td>
<td>Nifedipine</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
</tr>
<tr>
<td>Immunosuppressive agents</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td></td>
<td>FK-506 (tacrolimus)</td>
</tr>
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Table 1.2 Pgp modulators

<table>
<thead>
<tr>
<th>Class</th>
<th>Modulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-generation</td>
<td>Verapamil</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>Second-generation</td>
<td>PSC-833 (valspodar)</td>
</tr>
<tr>
<td></td>
<td>VX-710 (biricodar)</td>
</tr>
<tr>
<td>Third-generation</td>
<td>LY335979 (zosuquidar)</td>
</tr>
<tr>
<td></td>
<td>OC144-093 (ontogen)</td>
</tr>
<tr>
<td></td>
<td>XR9576 (tariquidar)</td>
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<tr>
<td></td>
<td>GF120918 (elacridar)</td>
</tr>
<tr>
<td>Others</td>
<td>Curcuminoids</td>
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<tr>
<td></td>
<td>Flavonoids</td>
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<tr>
<td></td>
<td>Disulfiram</td>
</tr>
<tr>
<td></td>
<td>Cremaphor EL</td>
</tr>
<tr>
<td></td>
<td>Nonidet P40</td>
</tr>
<tr>
<td></td>
<td>Benzyl alcohol</td>
</tr>
</tbody>
</table>

Due to the critical impact of Pgp on pharmacokinetics and multidrug resistance, numerous studies tried to identify and develop potential Pgp modulators. Several generations of Pgp modulators have been discovered, synthesized, and tested; however, even the best third-generation inhibitors have largely failed in clinical trials (36). Therefore, understanding the molecular mechanisms of Pgp modulators may be useful for understanding why they do not work in a clinical setting.
1.3.2 Nature of the drug-binding pocket

Poly-specific drug binding by Pgp results in the failure of chemotherapeutic treatment in many diseases, especially cancers. Knowledge of the drug-binding pocket is important to identify substrates, and to define a target for modulators, so they can effectively bind to Pgp and inhibit MDR. Several crystal structures of ABC transporters have shown structural details, such as the ATP binding site, inward- and outward-facing conformations of the TMDs, and the size of the cavity inside the TMDs (see reference (37) for examples). Only one study has shown the location and orientation of a specific substrate bound in the TMDs of Pgp (28). However, we are still lacking knowledge of how different drugs are bound in the pocket, and their exact location.

There are many approaches to biochemically characterize the drug-binding site of Pgp, including photoaffinity labelling with reactive substrate analogues (38,39), and the effect of drugs on ATPase activity and transport rates (40,41). Previous studies in our lab determined the binding affinity for ATP and substrates, using both extrinsic (26) and intrinsic (9) fluorescence spectroscopy. These fluorescence approaches have been reviewed previously (42-44). The quenching of Trp fluorescence by binding of nucleotides, substrates, and modulators, can be used to measure the $K_d$ in the range 20 nM - 250 µM (42,44). The large differences in $K_d$ values observed for individual compounds suggested that Pgp substrate recognition can be defined in a quantitative way, and is not simply non-specific. In addition, the biphasic quenching of Trp fluorescence exhibited by many substrates suggested the possibility of the existence of two
drug-binding sites of different affinity (42). The mouse Pgp crystal structure (28), which showed two molecules of a substrate binding to the TMDs at the same time, strongly supports the existence of sub-sites in the binding pocket.

The existence of two drug-binding sites, the H- and R-sites, was first reported by Shapiro and Ling, based on the transport of rhodamine123 and Hoechst 33342 (H33342), which enhanced each other’s transport by positively cooperative interactions (45). Using fluorescence resonance energy transfer (FRET), our group has mapped the potential location of H- and R-sites in the pocket to the region of Pgp within the cytoplasmic leaflet of the lipid bilayer (46,47). However, the crystal structure of mouse Pgp showed cyclic peptides QZ59-RRR and QR59-SSS located in the upper part of the cavity inside the binding pocket, which appeared to be in a different location from the H- and R-sites.

A recent molecular docking study using the mouse Pgp crystal structure has identified three putative drug-binding sites (48). The location of the H- and R-sites determined in this study agreed with the location previously determined by FRET, while the location of QZ59-RRR and QZ59-SSS binding was determined for the first time to be at a modulator site (M-site), which may have an important role in impairing conformational changes during drug efflux. Additionally, the effect of a second drug on substrate efflux by Pgp was explained by the value of the binding energies of individual drugs in each sub-site. For example, Shapiro and Ling (45) showed the concentration-dependent effect of colchicine and daunorubicin, which increased and decreased rhodamine 123 efflux by Pgp,
respectively. In the case of colchicine, it showed high binding free energy at the R-site, thus direct competition with rhodamine 123 results in displacement of colchicine to an alternative site, which favours rhodamine 123 transport, and impairs H33342 transport. On the other hand, daunorubicin has low binding free energy and directly competes with rhodamine 123, resulting in decreased rhodamine 123 transport.

Biochemical experiments also suggest that two substrate molecules can bind inside the pocket simultaneously. The interaction of Pgp with 6-(dimethylamino)-2-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl perchlorate (LDS-751) and rhodamine 123, which are believed to bind to the R-site, has been studied by fluorescence spectroscopy (49). The dyes interacted with each other non-competitively, resulting in a mutual reduction of their binding affinity by 5-fold. These results indicate that the two substrates were bound to Pgp simultaneously. Another experiment using Cys-scanning mutagenesis was conducted to test whether Pgp can accommodate two substrates simultaneously (50). By adding the thiol-reactive substrate, Tris-(2-maleimidoethyl)amine (TMEA) together with a second substrate, verapamil, stimulation of TMEA cross-linking by the second substrate in the drug-binding pocket of mutant F343C(TM6)/V982C(TM12) was observed. The results showed that verapamil can enhance cross-linking between two Cys residues in the mutant Pgp by TMEA, likely by inducing a conformational change. This also indicated that Pgp is able to accommodate verapamil and TMEA simultaneously in different regions of the drug-binding pocket.
1.3.3 Drug transport by Pgp

The process of efflux transport requires that a substrate binds in the drug-binding pocket, and the power to pump out the substrate is generated by ATP hydrolysis in the NBDs. The drug translocation pathway of Pgp was summarized by Callaghan et al., who described four key steps (Figure 1.3) (51). It is generally accepted that a substrate first partitions into the lipid bilayer, then interacts with the TMDs of Pgp. The direct interaction between substrate dissolved in the lipid bilayer and Pgp led to the description of the transporter as a “hydrophobic vacuum cleaner” (52). It has been proposed that substrates enter the drug-binding pocket of Pgp through gates, which are formed by TM segments 2/11 and 5/8 at either end of the drug-binding pocket (53). The affinity of *C. elegans* Pgp for two drugs, actinomycin D and paclitaxel, is approximately 4,000- and 100-fold higher in the lipid bilayer than in detergent (29). This increase suggested that drug partitioning into the membrane prior to interaction with Pgp is important.

After substrates bind to the TMDs, a key step in the drug transport mechanism is “cross-talk” between the TMDs and NBDs, which results in a conformational change in Pgp. This phenomenon is observed as the stimulation of ATP hydrolysis by substrate binding, which is an important way to identify Pgp substrates, and key residues for binding of substrate in the TMDs (54-62). This idea is supported by experiments using fluorescence spectroscopy (26). By using 2-(4’-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), site-directed fluorescence labelling (SDFL) covalently modifies a Cys residue within each NBD.
Figure 1.3 Pgp transport cycle

The four key steps of the Pgp transport cycle have been proposed by Callaghan et al. (51), (1) Drug and ATP loading promote NBD dimerization; (2) TMD reorientation switches the protein from the high affinity inward-facing conformation to low affinity outward-facing conformation; (3) ATP hydrolysis and nucleotide release; (4) resetting of the Pgp conformation and separation of the NBDs.
as a probe (26). Addition of nucleotide or substrate leads to alteration of Pgp conformation, leading to quenching of the probe fluorescence, which can be monitored. This approach can also be used to determine the drug-binding affinity within the TMDs. Fitting the quenching data of MIANS-Pgp to a binding equation allows estimation of the drug-binding affinity, $K_d$ under equilibrium conditions, for a variety of Pgp substrates (26). The ability of substrates and modulators to compete for drug transport by Pgp in membrane vesicles and proteoliposomes is highly correlated with their $K_d$ value (63,64), emphasizing the link between binding and transport. A recent study used molecular modelling and docking to propose locations for the H- and R-sites inside the binding pocket, using the mouse Pgp crystal structure (48).

The existence of at least two sites in Pgp, which interact with and transport drugs with different preferences, was proposed by Shapiro and Ling (45). Two transport sub-sites were described, the H-site, which preferentially transports H33342, and the R-site, which preferentially transports rhodamine 123 and its derivatives (45). Two H-site drugs were observed to compete with each other, inhibiting each other’s transport. Two R-site drugs also inhibited each other’s transport. However, an H-site drug stimulated transport of an R-site drug, and vice versa. The specific location of the H- and R-transport sites is not currently known, although the approximate place where H- and R-site drugs bind has been determined by fluorescence spectroscopy (46,47).

In ABC proteins, several highly conserved regions play an important role in the catalytic cycle of nucleotide binding and hydrolysis, including the Walker A
The nucleotide-binding sites are formed by dimerization of the two NBDs from each half of Pgp. As demonstrated in the crystal structure of Sav1866 from *Staphylococcus aureus*, two molecules of a nucleotide derivative, adenosine-5’-(β,γ-imino)triphosphate (AMP-PNP), appeared bound at the interface of the two NBDs (Figure 1.4). The Walker A and Walker B motifs from one NBD cooperated with the ABC signature motif from the other NBD.

The ATP hydrolysis cycle has been explained by two major models. The “switch” model, proposed by Higgins and Linton (65), demonstrated the switching between open and closed conformation of the NBDs during the ATP hydrolysis cycle. The closed conformation is initiated by binding of ATP molecules to create the NBD sandwich dimer, while ATP hydrolysis by the closed conformation drives it back to the open conformation, where the NBDs are separated. The second model, the “constant-contact” model, was proposed by Jones and George (66,67). This model suggested that the NBDs remained in contact during the catalytic cycle. ATP hydrolysis allowed the nucleotide-binding site to open and exchange nucleotide in an alternating manner one site at a time. Another significant difference between the two models is the mechanism to drive the drug from the high affinity site (inward-facing conformation) to the low affinity site (outward-facing conformation). In the switch model, the dimerization of NBDs by binding of ATP leads to conformational change in the TMDs from inward-facing to outward-facing, and ATP hydrolysis resets this conformational change (65). In contrast, the constant-contact model utilizes ATP hydrolysis at each site for the
Figure 1.4 Structure of an ABC nucleotide sandwich dimer

The crystal structure of Sav1866 from *Staphylococcus aureus* (PDB: 2ONJ) illustrates the structure of a nucleotide sandwich dimer. The N-terminal NBD is displayed as a yellow ribbon, while the C-terminal NBD is displayed as a grey ribbon. Each nucleotide-binding site is formed by the Walker A (green ribbon) and Walker B (red ribbon) motifs from one NBD, together with the ABC signature motif (purple ribbon) from the other NBD. One molecule of the nucleotide (AMP-PNP) (blue sticks) appears in each half of the NBD sandwich.
conformational change, which drives drug efflux (68). Both models agreed on the “alternating catalytic sites” scheme proposed by Senior et al. (69), which proposes that only one nucleotide-binding site hydrolyzes ATP at a time in an alternating manner.

The existence of two drug transport pathways was proposed by Peter Chiba and co-workers (70). To address the existence of dual drug translocation pathways in the TMDs of Pgp, peptide fragments of Pgp photolabelled with drug were identified by high-resolution mass spectrometry. Two labelled regions were identified, and further used as a target for site-directed mutagenesis, to produce mutations within two regions individually. The transport activities for rhodamine 123, verapamil, vinblastine, and propafenone were reduced in both mutants (70). However, the transport activities for verapamil, vinblastine, and propafenone in one mutant were reduced much more than the other mutant, whereas the reverse was true for rhodamine 123. This observation suggested that there are two preferential translocation pathways, one for rhodamine 123, and one for verapamil, vinblastine, and propafenone, and these results correlate the preferred site with the reduction of transport activity. It is still not clear whether these two transport pathways correspond to the H- and R-sites, and how they relate to the sub-sites for binding of drugs in the pocket.

The transport activity of Pgp can be monitored using a real-time fluorescence assay with the substrates tetramethylrosamine (TMR), H33342 and LDS-751 (see Figure 1.5), as developed in our laboratory. By adding ATP to the system, drug transport is initiated, and a decline in substrate fluorescence
emission is observed (for substrates used in this study) (Figure 1.6). This approach is suitable for Pgp reconstituted into proteoliposomes, and yields an initial rate of transport (40,71,72).

1.4 Fluorescence techniques

1.4.1 Intrinsic fluorescence of Pgp

Trp, Tyr and Phe are three amino acids that show fluorescent properties; of these, Trp residues show the highest quantum yield. It has been reported that the TM regions of Pgp are rich in highly conserved aromatic amino acid residues (73). The sequence of hamster Abcb1a Pgp showed 11 Trp residues (see Figure 1.1). The N-terminal half contains 6 Trp residues: one in the N-terminal tail (W44), one in the first intracellular loop (W159), and three residues in TM 2 (W133), 4 (W229) and 5 (W312), respectively, and one in an extracellular loop (W209). Two Trp residues are found in the linker region between NBD1 and TM7 (W695 and W705). The C-terminal half of Pgp contains 3 Trp residues; one is located in an intracellular loop (W800), one in an extracellular loop (W852), and one in NBD2 (W1105).

The effect of specific ligands on Pgp conformation was investigated using Trp quenching with iodide ion (74). Results showed that in presence of MgATP, the quenching effect of iodide ion was drastically increased, suggesting that a conformational change had increased solvent accessibility of Trp residues in Pgp. Further study by the same group demonstrated that another quencher, acrylamide, quenched Trp fluorescence in different patterns, depending on which
Figure 1.5 Structure of some fluorescent Pgp transport substrates

The transport substrates used in this research are shown; TMR (R-site), LDS-751 (both sites), and H33342 (H-site).
Figure 1.6 Substrate transport by reconstituted Pgp in proteoliposomes

(A) A cartoon showing drug transport by reconstituted Pgp in proteoliposomes. (B) Substrate transport is measured by monitoring the change in its fluorescence in real time. Transport of H33342 was initiated by adding ATP at time zero. The fluorescence intensity of H33342 is decreased when Pgp expels the substrate out of the lipid bilayer, compared to the case where an inhibitor of ATP hydrolysis is added (vanadate). The initial rate of transport is measured from the slope of the linear section from 10-20 s following addition of ATP.
ligand bound to Pgp, suggesting different degrees of conformational changes.

A previous study in our lab reported the Trp fluorescence properties of Pgp. The fluorescence spectrum of Pgp indicates that Trp residues are located in a highly nonpolar environment compared to the soluble Trp analogue N-acetyltryptophan amide (NATA) ($\lambda_{em}^{Pgp} = 333$ nm, $\lambda_{em}^{NATA} = 356$ nm). Trp fluorescence quenching in a saturable manner was observed with many drugs, modulators, hydrophobic peptides, and nucleotides, suggesting that the Trp residues of Pgp are good intrinsic fluorescent probes for substrate binding affinity determination (Figure 1.7). Additionally, it has been reported that many compounds exhibit biphasic quenching, suggesting that Pgp can bind multiple substrates at the same time.

1.4.2 Site-directed fluorescence labelling of Pgp on Cys residues

SDFL is a very useful technique to address many questions in protein structure using fluorescence spectroscopy (75-78). SDFL can be used to determine the secondary structure of a protein and solvent accessibility (79), map proximity in proteins (76), determine conformational changes in protein structure (80), and establish membrane protein topology (81). The hamster Pgp sequence contains 7 Cys residues (see Figure 1.1). The N-terminal half contains 2 Cys residues, one in TM2 (C134) and one in the Walker A motif (C428). The C-terminal half contains 5 Cys residues, two in TMDs (C714/TM7, C953/TM11), one in the Walker A motif (C1071), and two in the NBD (C1122 and C1224). A previous study by our group used MIANS to specifically label the two Cys
Figure 1.7 Trp quenching of Pgp by vinblastine and cyclosporin A

Increasing concentrations of vinblastine and cyclosporin A both showed the effect of binding on the intrinsic Trp fluorescence of Pgp. The quenching curve is fitted to an equation for a single binding site, shown by the solid line. Modified from Figure 2 in (43).
residues in the NBDs of Pgp (26). The fluorescence quenching of MIANS was used to determine the binding affinity of nucleotides and substrates; however, the ATPase activity of MIANS-labelled Pgp was completely abolished. Bimane derivatives are promising SDFL probes which covalently react with endogenous Cys residues or Cys residues introduced into proteins using site-directed mutagenesis (77). Several bimane derivatives are available commercially, including monobromobimane, dibromobimane and (2-pyridyl)dithiobimane (PDT-bimane) (Figure 1.8). Moreover, the size of bimane derivatives is relatively small, so they have limited ability to perturb protein structure. A previous study using Cys-scanning mutagenesis of Pgp showed that a bimane derivative, dibromobimane, is a substrate for Cys-less Pgp, and interacts with the substrate-binding pocket (57). Therefore, bimanes are an excellent choice to label Cys residues in the TMDs of Pgp. The change in fluorescence emission of bimane can be used to characterize the local environment around the bimane binding site. The reaction of a novel bimane derivative, PDT-bimane, can be monitored spectroscopically in real time (77) by measuring the absorption of pyridine-2-thione ($\lambda_{\text{max}} = 343$ nm) which is released from the reaction, as shown in Figure 1.8. Also, PDT-bimane is covalently linked to Cys residues by a disulfide bond instead of a thioether bond like monobromobimane and dibromobimane. Thus the labelling can be reversed by using a reducing agent such as Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). With this fluorescent probe, the stoichiometry of labelling can be estimated by quantifying release of the bimane.
Figure 1.8 Structure and reaction of bimane derivatives and MIANS

The structure of MIANS and the bimane derivatives used in this study, including monobromobimane, dibromobimane and PDT-bimane, are shown. PDT-bimane reacts with Cys residues in proteins to form a bimane adduct, releasing pyridine-2-thione.
group following cleavage by reducing agents (77). To date, bimane labelling has not been used to study the structure and function of Pgp.

1.5 Rationale, hypothesis and objectives

To date, several studies have demonstrated the ability of Pgp to bind two unrelated substrates in the binding pocket simultaneously. Due to the lack of information from both biochemical and structural studies, the exact location of the substrate-binding sub-sites has not been determined and the principles of binding and transport of substrate are still not fully understood. Therefore, my research is focused on the binding pocket of the transporter, and its sites for binding of different drugs. My hypothesis is that the drug binding pocket of Pgp has sub-sites to accommodate different types of drugs simultaneously. When two drug molecules are bound, they may affect each other’s binding properties and transport, depending on the degree of overlap between the sub-sites where they are located.

The goals of this project were to better understand the properties of the substrate-binding pocket of Pgp, especially drug-drug interactions inside the pocket. To avoid the complication of multiple reversible drug equilibria, a selected Pgp substrate (bimane) was used to covalently label the binding pocket of Pgp, to mimic the physical interaction of a substrate with the transporter. This allowed the binding and transport of a second substrate to be measured using a panel of fluorescence assays.
Chapter 2

Materials and methods
2.1 Materials

NATA, TCEP, Folin & Ciocalteu’s phenol reagent, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic 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, Precision Plus Protein Kaleidoscope standards (10-250 kDa) and Bradford reagent were purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin (BSA) (crystallized) was obtained from Roche Diagnostics (Laval, QC). PDT-bimane was purchased from Cedarlane (Burlington, ON). H33342, LDS-751, TMR, and MIANS were purchased from Life Technologies (Burlington, ON). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from MP Biomedicals (Solon, OH). The eStain® Protein Staining Pads (Coomassie blue G-250) and the eStain® Protein Staining System were purchased from GenScript (Piscataway, NJ). Egg phosphatidylcholine (PC) was obtained from Avanti (Alabaster, AL).

2.2 Pgp purification

A differential CHAPS extraction for Pgp isolation from the plasma membrane of the multidrug-resistant Chinese hamster ovary cell line CHB30 was performed as previously described (9,82). All the buffers used in the purification steps were prepared using 20 mM HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.4). Dithioerythritol (DTE) was omitted from all of the buffers. Plasma membrane (approximately 40-60 mg) was
thawed and centrifuged at 41,000 rpm (173,000 xg) for 30 min at 4°C. The supernatant was discarded and the plasma membrane pellet was resuspended in 1 mL of cold extraction buffer I (15 mM CHAPS/20 mM HEPES buffer) for each 20 mg of plasma membrane protein, using a 1 mL syringe with a 21G1 needle to gently mix. The suspension was incubated on ice with periodic swirling for 30 min and centrifuged at 41,000 rpm (173,000 xg) at 4°C for 20 min. The supernatant (S1) was discarded and the plasma membrane pellet was further resuspended in 1 mL of cold extraction buffer II (45 mM CHAPS/20 mM HEPES buffer) for every 10 mg of plasma membrane protein, using a 1 mL syringe with a Sub-Q 26G5/8 needle to gently mix. The suspension was incubated for 30 min on a Nutator at 4°C, and then centrifuged at 15,000 xg for 15 min at 4°C. The supernatant (S2) was transferred to another tube and further run through a column of Con A-Sepharose 4B (d = 1.5 cm, L = 20 cm, V = 35 mL) which was pre-equilibrated with ~60-70 mL of 2 mM CHAPS/20 mM HEPES buffer. The S2 fraction was loaded onto the column and eluted with cold 2 mM CHAPS/20 mM HEPES buffer. The samples eluted from the column were collected in ~1 mL/fraction by the fraction collector. All the separation steps were performed at 4°C. The protein content and ATPase activity in each fraction were determined by Bradford assay (see section 2.3) and ATPase assay, respectively (see section 2.4). Fractions with high protein content and ATPase activity were pooled and stored at -70°C for further experiments.
2.3 Protein assay

Protein concentrations were determined at several protein purification steps, during Pgp-bimane adduct preparation, and in reconstituted proteoliposomes. Two protein assays, the Bradford assay and the modified Lowry assay, were used in this study. Bradford assay was used to determine the amount of Pgp during purification and relative ATPase activity determination, as previously described (83). Briefly, standard BSA (from 0-3 µg) and protein samples, which were individually adjusted with superQ water to a total volume of 20 µL, were transferred into a 96-well plate. A 250-µL aliquot of Bradford reagent was added to each triplicate standard and sample, which were allowed to sit at room temperature for 10 min. The absorbance of each well at 595 nm was measured with a microplate reader. All standards and samples were measured in triplicate.

The modified Lowry assay was typically used for stoichiometry of labelling determination and protein determination in reconstituted proteoliposomes, as previously described (84). Briefly, standard BSA (0-20 µg) and protein samples, individually adjusted with superQ water to a total volume of 100 µL, were precipitated in a total volume of 1.3 mL with 6% (w/v) trichloroacetic acid (TCA) in the presence of 1% (w/v) deoxycholate (DOC). All samples were centrifuged in a microcentrifuge at 10,400 rpm (10,000 xg) for 20 min; the supernatant was discarded. Protein pellets were resuspended in 300 µL superQ water and 300 µL of solution A (a mixture of equal volumes of 10% (w/v) SDS, 0.8 M NaOH and CTC solution (10% (w/v) sodium carbonate, 0.1% (w/v) CuSO₄ and 0.2% (w/v)
potassium tartrate). The suspensions were incubated at room temperature for 10 min, then 150 µL of solution B (0.4 N Folin & Ciocalteu’s phenol reagent) was added and they were incubated at room temperature for another 45 min. Each sample was transferred to a 96-well plate, and the absorbance at 750 nm was measured using a microplate reader. All standards and samples were measured in duplicate.

2.4 ATPase assay

Specific ATPase activity of Pgp was determined by ATPase assay, a colourimetric assay that measures inorganic phosphate release from ATP (85). Briefly, the reaction was performed in a total volume of 100 µL in 96-well plates. Purified Pgp and various concentrations of standard inorganic phosphate (0-20 µg) were prepared in 90 µL total volume in a 96-well plate. The reaction was started by adding 10 µL of 10 mM ATP (final concentration 1 mM), and the plate was incubated at 37°C. After 20 min incubation, 100 µL of stop reagent (0.5% (w/v) ammonium molybdate, 6% (w/v) SDS, 3% (w/v) ascorbic acid) was added into each well, followed by incubation at room temperature for 5 min. A 100-µL aliquot of 2% (w/v) sodium citrate/ 2% (v/v) acetic acid/ 2% (w/v) sodium acetate was added to each well. The samples were allowed to sit for 20 min to let the colour develop, and the absorbance at 750 nm was read on a plate reader. All the standards and samples were measured in triplicate.

In order to see whether ATPase activity of bimane-labelled Pgp can be regenerated by TCEP, the ATPase activity of bimane-labelled Pgp was carried
out using some modifications. Duplicates of Pgp samples (approximately 0.5 µg), including Pgp, Pgp-bimane(-ADP) and Pgp-bimane(+ADP), were prepared with 2 mM CHAPS/20 mM HEPES buffer to make a total volume of 90 µL in a 96-well plate. Another set of these samples was prepared as described above, with the addition of 0.5 µL of 0.1 M TCEP (final concentration ~0.5 mM), then incubated at room temperature for 30 min. The rest of the assay was performed as described above.

2.5 SDS-PAGE

The purity of Pgp, and the covalent binding of fluorescence probes to the protein, were determined by SDS-PAGE. Protein samples (2-5 µg) were mixed with loading buffer at a volume ratio of 4:1, then a total volume of 20 µL of each sample and Precision Plus Protein Kaleidoscope standards (10-250 kDa) were loaded into each well. Electrophoresis was carried out at 300 V for approximately 90 min. For purity estimation, the gel was stained by eStain® Protein Staining Pads (Coomassie blue G-250) with the eStain® Protein Staining System. Destaining was carried out using 50% (v/v) methanol/ 7% (v/v) acetic acid. The gel was incubated in destaining solution overnight on a rocking table at room temperature. An image of the stained gel was captured using a Fluorchem™ 8900 imaging system. For visualizing covalent binding of fluorescence probe to Pgp, the gel was viewed and imaged under UV light (λ<sub>ex</sub> = 302 nm, 595 nm cut-on emission filter) prior to Coomassie staining.
2.6 Preparation of the Pgp-bimane adduct

2.6.1 Site-directed PDT-bimane labelling of Pgp

Preparation of Pgp-bimane adducts was performed under light protection at room temperature. A total reaction volume of 1 mL was prepared in a 1.5-mL microcentrifuge tube, containing 900 µL of purified Pgp (~350-400 µg) in 2 mM CHAPS/20 mM HEPES buffer, pH 7.4, 10 µL of 50 mM PDT-bimane in acetonitrile (the volume of PDT-bimane was minimized to make sure that acetonitrile is no more than 1% (v/v)) to make a final concentration of 0.5 mM and 2 mM CHAPS/20 mM HEPES buffer, to make up the total volume. The reaction was started by adding PDT-bimane to the Pgp solution and mixing the suspension with a 100-µL pipette, followed by 30-min incubation time. The excess PDT-bimane was removed at 4°C on a Bio-Rad Econo10-DG gel filtration column (exclusion size 6 kDa) which was pre-equilibrated with 2 mM CHAPS/20 mM HEPES buffer. The eluent was collected using a fraction collector to collect 10 drops (250 µL) for each fraction, which were analyzed for protein content and bimane fluorescence ($\lambda_{ex} = 381$ nm, $\lambda_{em} = 478$ nm). Typically, the labelled Pgp fractions that had a protein content higher than 0.2 mg/mL were pooled for drug transport experiments, while the other fractions with a protein concentration higher than 0.05 mg/mL were collected separately for drug binding experiments. Pgp was labelled under two conditions; in the absence of ADP (to give Pgp-bimane(-ADP)) and in the presence of 5 mM ADP (to give Pgp-bimane(+ADP)).
Pgp ATPase activity was completely abolished by PDT-bimane labelling. To preserve the ATPase activity, purified Pgp was preincubated with ADP on ice during the PDT-bimane labelling step. A 900-µL sample of purified Pgp in 2 mM CHAPS/20 mM HEPES buffer was mixed with 50 µL of 100 mM ADP (final concentration 5 mM) and preincubated on ice for 5 min, then room temperature for 5 min.

2.6.2 PDT-bimane labelling optimization

As PDT-bimane labelling abolishes the ATPase activity of Pgp, the specific ATPase activity of the protein was used to monitor the labelling reaction. To measure the concentration-dependent effect of PDT-bimane on Pgp ATPase activity, the ATPase assay (see section 2.4) was adapted by adding various concentrations of PDT-bimane to 0.5 µg of purified Pgp in 2 mM CHAPS/20 mM HEPES buffer. The reaction was started by adding ATP to each well immediately, and the 96-well plate was incubated for 5 min at room temperature. Each concentration of PDT-bimane was performed in duplicate. The ATPase assay was then completed as described previously.

To determine the time-dependent effect of PDT-bimane labelling on ATPase activity, an adapted ATPase assay was performed. In a total volume of 90 µL, a 0.1 mM final concentration of PDT-bimane was added to 0.5 µg of purified Pgp in 2 mM CHAPS/20 mM HEPES buffer at different time intervals varying from 60-0 min. ATP was added immediately to all wells after the time zero sample, and the 96-well plate was incubated at 37°C for 5 min. Each
incubation time was performed in duplicate. The ATPase assay was then completed as described previously.

2.6.3 Stoichiometry of PDT-bimane labelling

The stoichiometry of PDT-bimane labelling was calculated from the ratio between measured bound bimane and protein concentration. To determine the bound PDT-bimane, the calculation was done by subtracting the free PDT-bimane in the sample from the total PDT-bimane. A duplicate sample of 100 µL of Pgp-bimane was prepared differently for free PDT-bimane and total PDT-bimane measurement. For determination of free PDT-bimane, a 50-µL (~10-20 µg) Pgp-bimane sample with 50 µL of 2 mM CHAPS/20 mM HEPES buffer was precipitated at room temperature by adding 20 µL of 36% (w/v) TCA, followed by mixing with a pipette. The sample was incubated for 5 min, then centrifuged with a microcentrifuge at 10,400 rpm (10,000 xg) for 20 min. The protein pellet was discarded; to the supernatant 0.7 µL of 0.1 M TCEP was added and the sample was incubated for 30 min at room temperature. TCEP reduces the labelled Pgp and release free bimane into the solution. For determination of total PDT-bimane, the same volume of Pgp-bimane was treated with TCEP for 30 min before it was precipitated with TCA, using the same conditions as for free PDT-bimane. A standard curve of bimane fluorescence was prepared by treating standard PDT-bimane (0-10 µM) with 0.1 M TCEP and 6% (w/v) TCA in the same way as for the total PDT-bimane sample. The fluorescence at 478 nm was measured at 20°C following excitation at 381 nm using a 100 µL quartz cuvette (0.5-cm path length), for the standards and Pgp-bimane. All the samples were read
approximately 1 hour after TCEP treatment. The amount of bound PDT-bimane was calculated by subtracting the free PDT-bimane from the total PDT-bimane, using the same sample size of Pgp-bimane. The protein concentration of the Pgp-bimane sample was determined by a modified Lowry assay as described above (section 2.3). The stoichiometry of PDT-bimane labelling was calculated as the molar concentration of bound PDT-bimane divided by the molar protein concentration.

A control for PDT-bimane labelling was performed with a purified sample of a single Cys mutant protein, colicin E1 S354C, which was provided by the laboratory of Dr. A.R. Merrill at the University of Guelph. The expression and purification of colicin E1 proteins containing a single Cys residue, including the S354C mutant, was described previously (86). Colicin E1 S354C was used for PDT-bimane labelling at the same concentration as Pgp (0.4 µg/mL) by diluting with 2 mM CHAPS/20 mM HEPES buffer. The stoichiometry determination was performed as described above.

2.6.4 Optimization of bimane cleavage by TCEP

The cleavage of bimane from free PDT-bimane and bimane-labelled Pgp using TCEP was optimized in order to obtain an accurate estimate number for the stoichiometry of labelling. For cleavage of free PDT-bimane, 100 µL of 2 µM PDT-bimane in the presence of 6% (w/v) TCA was transferred to a 100 µL cuvette (0.5-cm path length), then 0.5 µL of 0.1 M TCEP (0.5 mM final concentration) was added. The fluorescence intensity ($\lambda_{\text{ex}} = 381$ nm, $\lambda_{\text{em}} = 478$...
nm) was measured after 5, 10, 15, 20 and 30 min at 20°C. For cleavage of bimane from Pgp, 100 µL of 0.1 mg/mL Pgp-bimane (~10 µg) was treated with 0.5 µL of 0.1 M TCEP (0.5 mM final concentration) for 5, 10, 15, 20 and 30 min, then 20 µL of 36% (w/v) TCA was added (6% (w/v) final concentration). The protein sample was centrifuged with a microcentrifuge at 10,400 rpm (10,000 xg) for 20 min. The supernatant was transferred to a 100-µL quartz cuvette, and the fluorescence intensity (λ<sub>ex</sub> = 381 nm, λ<sub>em</sub> = 478 nm) was measured at 20°C.

### 2.6.5 pH dependence of bimane fluorescence

A 100 µM reduced PDT-bimane stock solution was prepared by mixing equal volumes of 200 µM PDT-bimane with 3 mM TCEP, then incubating for 30 min at room temperature in the dark. Various concentrations of TCA from 0-72% (w/v) were prepared in 2 mM CHAPS/20 mM HEPES buffer. The fluorescence intensity of bimane (λ<sub>ex</sub> = 381 nm, λ<sub>em</sub> = 478 nm) was measured for a mixture of 1 µL of reduced PDT-bimane stock solution with 99 µL of 2 mM CHAPS/20 mM HEPES buffer containing various TCA concentrations, using a 100-µL quartz cuvette (0.5-cm path length). All fluorescence measurements were carried out at 20°C. The data were corrected by subtracting the fluorescence of 2 mM CHAPS/20 mM HEPES buffer containing TCA at the same concentrations.

### 2.7 Characterization of Pgp-bimane adducts

All the fluorescence measurements were carried out using a PTI QuantaMaster QM-8/2005 with Peltier temperature control. The instrument temperature was preset to 20°C for all experiments unless indicated, and the
excitation and emission bandpasses were set to 3 nm and 2 nm, respectively, unless otherwise stated. Fluorescence measurements were carried out in a 100 µL quartz cuvette (0.5-cm path length).

### 2.7.1 Fluorescence spectra of Pgp-bimane

The fluorescence properties of Pgp-bimane were characterized using three averaged excitation and emission scans. To correct the background, an identical scan was carried out using 2 mM CHAPS/20 mM HEPES buffer and unlabelled Pgp in the same buffer. These were subtracted from the free PDT-bimane spectrum and the Pgp-bimane spectrum, respectively.

### 2.7.2 Double labelling of Pgp-bimane with MIANS

To prove that preincubation of Pgp with ADP can protect a Cys residue in each NBD from reaction with PDT-bimane, a second labelling step with MIANS was performed. MIANS labelling can be monitored in real time by measuring the increase in fluorescence emission at 420 nm ($\lambda_{ex} = 320$ nm), since unreacted free MIANS has no fluorescence. MIANS labelling of Pgp-bimane(+ADP) and Pgp-bimane(-ADP) was performed in a 100 µL quartz cuvette (0.5-cm path length) at room temperature, in the presence of either no ADP or 5 mM ADP. A 90-µL aliquot of labelled Pgp (~2.5 µg) was mixed with 5 µL of 100 mM ADP (for ADP protection) or 2 mM CHAPS/20 mM HEPES buffer (for no ADP protection), and preincubated for 5 min. Fluorescence emission was recorded in real time at 420 nm following 320 nm excitation for 100 s, then 5 µL of 80 µM MIANS in ethanol was added to make a final concentration of 4 µM and fluorescence
emission was recorded for an additional 200 s. The background was corrected by subtracting the fluorescence emission of 4 µM MIANS in 2 mM CHAPS/20 mM HEPES buffer at room temperature.

2.7.3 Determination of substrate binding affinity

The binding of various drugs to Pgp, Pgp-bimane(-ADP) and Pgp-bimane(+ADP) were determined by measuring either Trp quenching or bimane quenching. A 80-µL aliquot of purified Pgp (50 µg/mL) in 2 mM CHAPS/20 mM HEPES buffer was added to a quartz microcuvette (0.5-cm path length) and allowed to equilibrate for 5 min in the fluorimeter at 20°C. Drug aliquots of 0.8 µL in DMSO were added, then the sample was mixed 7 times using a micropipette. The steady-state Trp fluorescence emission was measured at 330 nm with 290 nm excitation, and steady-state bimane fluorescence emission was measured at 478 nm with 381 nm excitation. The sample was titrated with 0.8-µL aliquots of DMSO alone to ensure that it did not affect Trp or bimane fluorescence. To correct for non-specific quenching, an identical drug titration was performed with 20 µM NATA and 4 µM PDT-bimane.

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

$$F_{icorr} = (F_i - B) \left( \frac{V_i}{V_0} \right) \left[ \frac{(F_{0NATA} - B) \left( \frac{V_i}{V_0} \right)}{(F_{0NATA} - B) \left( \frac{V_i}{V_0} \right)} \right]$$
where \( F_{\text{corr}} \) is the corrected fluorescence intensity after the drug aliquot was added, \( B \) is the buffer fluorescence intensity, \( V_0 \) is the sample initial volume, \( V_i \) is the sample final volume at any point in the titration, \( F_{0\text{NATA}} \) is the initial NATA fluorescence intensity, and \( F_{\text{inATA}} \) is NATA fluorescence intensity at any point in the titration. All the measured fluorescence intensity data were normalized and plotted against drug concentration, and fitted to the following hyperbolic equation:

\[
\Delta F = \frac{\Delta F_{\text{max}} \times [S]}{K_d + [S]}
\]

where the \( \Delta F \) represents the change in fluorescence intensity following addition of substrate at a concentration \([S]\). Fitting was performed by nonlinear regression with the Marquardt-Levenberg algorithm using SigmaPlot; values of \( K_d \) and the maximum percent fluorescence quenching \( \Delta F_{\text{max}} \) were extracted.

2.7.4 CD spectroscopy

The melting points of Pgp and bimane-labelled Pgp were determined by CD spectroscopy. A 400-µL aliquot of protein (70 µg/mL) in 2 mM CHAPS/20 mM HEPES buffer was transferred into a cuvette (0.3-cm path length). The CD spectrum was recorded along an increasing temperature gradient from 30-70ºC. The ellipticity values at 222 nm were normalized and fitted to a 4-parameter sigmoidal curve using SigmaPlot. The \( T_m \) value was defined as the inflection point.
2.8 Drug transport

2.8.1 Reconstitution of Pgp into proteoliposomes

Pgp was reconstituted into egg PC proteoliposomes using a modification of a method previously developed in our lab (87). Briefly, 45 µL of 100 mg/mL egg PC prepared in chloroform-methanol was dried under a stream of nitrogen for 5 min and placed in a pump vacuum desiccator for 45 min to remove all remaining solvent. Dried lipid was redissolved in 100 µL of 500 mM CHAPS/20 mM HEPES buffer, vortexed, and incubated at 37°C for 10 min. A 1000-µL total volume of unlabelled Pgp or labelled Pgp, which were adjusted to the same concentration prior to reconstitution, was added directly into the lipid solution, and mixed ~20 times with a 26%G needle. The mixture was incubated on a Nutator at 4°C for 45 min and then loaded on a Sephadex G-50 gel filtration column (d = 1.0 cm, L = 20 cm, V = 20 mL), which had been pre-equilibrated with 20 mM HEPES buffer, containing no CHAPS. The sample was eluted with 20 mM HEPES buffer and 10 drops (0.25 µL/fraction) were collected in each fraction. Collected fractions were analyzed for protein content by Bradford assay. The fractions that contained 0.2 µg/µL Pgp or higher were collected and pooled together for drug transport experiments. The final protein concentration of the pooled fractions was confirmed by Lowry assay. All proteoliposomes were kept on ice and used for experiments the same day.
2.8.2 PDT-bimane inhibition of drug transport by unlabelled Pgp

Drug transport was measured using a real-time fluorescence assay, as described previously (42,71,72), with some modifications. All fluorescence measurements using egg PC proteoliposomes were carried out at 20°C. The various concentrations of PDT-bimane were prepared in acetonitrile. A 71-µL aliquot of unlabelled Pgp proteoliposomes was added to 4 µL of 20 µM transport substrate (TMR or H33342) to make a final concentration of 1 µM for all substrates and preincubated for 30 min on ice in the dark. The mixture was transferred into a 100-µL quartz microcuvette with a path length of 0.5 cm, then the transport assay was started by adding 4 µL of 20 mM ATP (final concentration 1 mM), followed by 1 µL of prepared PDT-bimane (final concentrations 0 – 100 µM). Fluorescence emission of TMR, H33342, and LDS-751 was collected at 575, 450 and 650 nm, after excitation at 550, 355, and 555 nm, respectively. The initial rate of drug transport was obtained by fitting the linear portion of the curve of the fluorescence emission versus time, over a period of 20 s (5 – 25 s after initiation of transport). The initial rate of drug transport was plotted against PDT-bimane concentration, and fitted using SigmaPlot to a hyperbolic equation:

\[
v = \frac{V_{\text{max}}[S]}{IC_{50} + [S]}
\]
where $v$ is the initial rate of substrate transport at a certain concentration of PDT-bimane, $[S]$. The IC$_{50}$ value at which transport was inhibited by 50% was extracted using SigmaPlot.

### 2.8.3 Drug transport by Pgp-bimane and unlabelled Pgp

Fluorescence emission measurements were carried out at 20°C, with emission and excitation bandpasses at 4 nm. Various concentrations of TMR, H33342, and LDS-751 were prepared in DMSO. Prior to measuring drug transport, the protein concentration of proteoliposomes for both unlabelled Pgp and Pgp-bimane was adjusted with 20 mM HEPES buffer to make them equal (~0.20 – 0.25 mg/mL). To a 72-µL aliquot of unlabelled Pgp proteoliposomes and Pgp-bimane proteoliposomes was added 4 µL of various concentrations of transport substrates, followed by incubation on ice for 30 min in the dark. Before the transport assay was performed, all samples was preincubated at 20°C for 5 min prior to transfer to a 100-µL quartz microcuvette. Drug transport was started by adding 4 µL of 20 mM ATP (1 mM final concentration), then the fluorescence emission was collected at the appropriate wavelength (section 2.8.2). The initial rate of transport was determined as described above (section 2.8.2). The initial rate of drug transport was plotted against the drug concentration, the data were fitted to the Michaelis-Menten equation, and $K_M$ and $V_{max}$ were extracted using SigmaPlot.

$$v = \frac{V_{max}[S]}{K_M + [S]}$$
Chapter 3

Site-directed PDT-bimane labelling of Pgp
3.1 Introduction

The amino acid sequence of Chinese hamster Pgp previously reported by Devine et al. (88) showed that there are 7 Cys residues, two in the N-terminal half and the rest in the C-terminal half. The proposed topology of Chinese hamster Pgp indicates that 3 Cys residues are located in the TMDs, 2 Cys residues in the Walker A motifs of each half, and 2 Cys residues in the cytoplasmic regions of the protein (40). Dibromobimane was previously identified to be a potent stimulator of ATPase activity in Cys-less human Pgp (54), suggesting that it is a substrate, and that it interacts with the drug-binding pocket. Dibromobimane was also used with single Cys-scanning mutagenesis to identify residues within the drug-binding domain of Pgp (57).

The ATPase activity of Pgp can be affected by both non-covalent binding of PDT-bimane and covalent labelling of Cys residues. Numerous studies have previously shown the effect of covalently modifying the Cys residues in the Walker A motifs, which are highly conserved regions in the NBDs. A study on hamster Pgp using N-ethylmaleimide (NEM), a sulfhydryl-reactive agent, was first reported by Al-Shawi et al. (89). They found that NEM inactivated Pgp ATPase activity by reacting at two sites, one in each half of the protein. A later study by Loo and Clarke showed that NEM covalently labelled the two Cys residues in the NBDs and inhibited the ATPase activity of human Pgp (27). By constructing and expressing a Cys-less Pgp protein and reintroducing a Cys residue at a specific location, each Cys residue in Pgp can be examined individually. They demonstrated that the two Cys residues in the NBDs, C431 and C1074, are
responsible for the NEM sensitivity of human Pgp, and this inactivation can be prevented by binding of ATP. This also agrees with the results from our group, in which the ATPase activity of hamster Pgp was completely inhibited by labelling with the thiol-reactive fluorescent probe, MIANS (26). Thus, it was expected that the thiol reactivity of PDT-bimane would result in modification of the Walker A Cys residues, and loss of ATPase activity. Cys-less Pgp was also used to investigate the effect of dibromobimane binding on ATPase activity in the absence of a covalent reaction (54). Results showed that dibromobimane binding can stimulate the ATPase activity of human Pgp by 8.2-fold, while dibromobimane conjugated with free Cys amino acid showed no effect. However, how dibromobimane binds to the Pgp binding pocket is still unclear.

The objective in this chapter was to label purified Pgp with PDT-bimane on Cys residues so that it still retains ATPase activity and drug transport function. To accomplish this, the labelling process was optimized, and the effectiveness, efficiency, and target Cys residues of PDT-bimane were determined. Bimane-labelled Pgp was characterized spectroscopically and functionally with respect to its ATPase activity, and was used for further study.

### 3.2 Effect of PDT-bimane on Pgp ATPase activity

Many studies have observed the interaction of substrates with Pgp through their effect on its ATPase activity (90). The effect of bimane derivatives (5 \( \mu \)M – 1 mM concentration range), including monobromobimane, dibromobimane, and PDT-bimane, on ATPase activity of purified Pgp in 2 mM
CHAPS/20 mM HEPES buffer was monitored. The ATPase assay was initiated immediately after PDT-bimane was added to Pgp, using 5 min and 20 min assay times. Since the covalent labelling reaction will proceed during the incubation, the reduced 5 min assay time was anticipated to give a more accurate picture of the effect of bimanes on ATPase activity. Figure 3.1A shows the ATPase activity of Pgp in the presence of various concentrations of PDT-bimane for the two assay times. The ATPase activity of Pgp was completely abolished at PDT-bimane concentrations above 0.3 mM, suggesting that the covalent labelling reaction is relatively fast. By using a lower range of PDT-bimane concentrations, more details of the PDT-bimane sensitivity of Pgp can be seen in Figure 3.1B. In contrast, the other two bimane derivatives, monobromobimane and dibromobimane, only partially inhibited ATPase activity of purified Pgp. Maximum inhibition occurred at 0.5 – 0.7 mM and 0.5 – 1 mM for monobromobimane (Figure 3.2) and dibromobimane (Figure 3.3), respectively. The maximum inhibition was ~50% for both of them. A PDT-bimane concentration of 0.1 mM, where Pgp still retained partial ATPase activity, was further used to determine the time-dependence of labelling. Purified Pgp was incubated with 0.1 mM PDT-bimane for various times from 0 – 60 min, then the ATPase activity was determined immediately with a 5 min assay time. The results in Figure 3.4 indicate that it took less than 10 min to almost completely abolish the ATPase activity of Pgp, suggesting that the labelling reaction was completed within this time. For stoichiometric labelling of Pgp with PDT-bimane, a higher concentration
Figure 3.1 Concentration dependence of PDT-bimane inhibition of ATPase activity of purified Pgp

A) Purified Pgp in 2 mM CHAPS/20 mM HEPES buffer (0.5 µg) was mixed with different concentrations of PDT-bimane in the range 0 – 1 mM and ATP was added immediately. After 5 min or 20 min at 37°C, the assay was stopped and ATP hydrolysis determined (section 2.4). B) Purified Pgp was mixed with PDT-bimane in the concentration range 0 – 100 µM, and the ATPase assay was carried out as in A, with a 5 min assay time. Data points are presented as % control with no PDT-bimane added and represent 3 replicate measurements (mean ± SEM).
Figure 3.2  Concentration dependence of monobromobimane inhibition of ATPase activity of purified Pgp

Purified Pgp in 2 mM CHAPS/20 mM HEPES buffer (0.5 µg) was mixed with different concentrations of monobromobimane in the range 0 – 1 mM and ATPase activity was measured using a 5 min or 20 min assay time at 37°C. Data points are presented as % control with no monobromobimane added and represent 3 replicate measurements (mean ± SEM).
Figure 3.3  Concentration dependence of dibromobimane inhibition of ATPase activity of purified Pgp

Purified Pgp in 2 mM CHAPS/20 mM HEPES buffer (0.5 µg) was mixed with different concentrations of dibromobimane in the range 0 – 1 mM and ATPase activity was measured using a 5 min or 20 min assay time at 37°C. Data points are presented as % control with no dibromobimane added and represent 3 replicate measurements (mean ± SEM).
Figure 3.4  Time dependence of PDT-bimane inhibition of ATPase activity of purified Pgp

Purified Pgp in 2 mM CHAPS/20 mM HEPES buffer (0.5 µg) was incubated with 0.1 mM PDT-bimane for different times (0 – 60 min) at 37°C, and 5 min ATPase assay was performed immediately. Data points are presented as % control with no PDT-bimane added and represent duplicate measurements (mean ± range). Where error bars are not visible, they are contained within the symbols.
(0.5 mM) and longer time of incubation (30 min) were selected to ensure completion of the labelling reaction.

### 3.3 PDT-bimane labelling of Pgp

As PDT-bimane is a light-sensitive reagent, all the labelling steps were done under light protection to minimize photobleaching. Labelling conditions were selected accordingly to previous characterization (see section 3.2). The reaction mixture was run through an Econo 10-DG gel filtration column, and both protein content and bimane fluorescence were monitored in the fractions (Figure 3.5). Two or three peak fractions were collected and pooled according to the separation results. After passing bimane-labelled Pgp through the column, it was considered to be separated from free PDT-bimane. Pgp labelled by PDT-bimane under these conditions showed a 100% loss of ATPase activity.

### 3.4 Optimization of PDT-bimane labelling

The objective of the optimization process was to find the optimal conditions for preparation of a homogeneous Pgp drug adduct that still has transport function. Covalent labelling of the NBD Cys residues with PDT-bimane likely leads to complete loss of ATPase (and transport) activity. The goal was to prevent labelling of these residues. Previous studies in our group showed that the two Cys residues in the NBDs were labelled with MIANS, and that labelling can be prevented by binding ATP to Pgp prior to the labelling step (26). This observation also agrees with the study by Loo and Clark, who used ATP to prevent NEM inactivation (27). The initial labelling condition (0.1 mM PDT-
**Figure 3.5  Separation of labelled Pgp-bimane from free PDT-bimane by gel filtration chromatography**

After labelling Pgp with PDT-bimane, excess free PDT-bimane was separated by gel filtration chromatography at 4°C. Individual fractions of 0.25 mL were collected, followed by Bradford assay and bimane fluorescence measurement to monitor the separation.
bimane with 30 min incubation time at 22°C in the dark, in the absence of ADP) was selected to ensure that all the available Cys residues were labelled. The stoichiometry of PDT-bimane labelling (see section 2.6.3) and the ATPase activity of Pgp labelled under these conditions, designated as Pgp-bimane(-ADP), were determined. Labelling with PDT-bimane was then performed as described above with the additional step of incubating purified Pgp with 5 mM ADP for 10 min on ice prior to the addition of PDT-bimane. The stoichiometry of labelling and ATPase activity of Pgp labelled under these conditions, designated as Pgp-bimane(+ADP), were determined, using the same amount of labelled Pgp. ADP was shown to preserve the ATPase activity of Pgp-bimane(+ADP), with 53 ± 7% activity retained compared to unlabelled Pgp, while Pgp-bimane(-ADP) showed no ATPase activity (Figure 3.6). This result suggests that preincubation with ADP protects certain Cys residues from being labelled by PDT-bimane, and in consequence, retains ATPase activity of the Pgp-bimane adduct. Interestingly, when either Pgp-bimane(-ADP) or Pgp-bimane(+ADP) was treated with 0.1 mM TCEP for 10 min to cleave covalently bound bimane, ATPase activity was restored up to 90% compared to unlabelled Pgp treated with TCEP under the same conditions. This observation confirms that modification of some Cys residues is responsible for loss of ATPase activity.

3.5 Stoichiometry of PDT-bimane labelling

Determination of the efficiency of labelling with bimane derivatives, including monobromobimane and dibromobimane, was first carried out by Kosower et al. (91). Their study showed the effectiveness of bimane labelling by
Figure 3.6 Protection of Pgp ATPase activity during bimane labelling by addition of ADP

Both Pgp-bimane(-ADP) and Pgp-bimane(+ADP) were assayed for ATPase activity remaining after the labelling process. The ATPase activity of both types of bimane-labelled Pgp was also measured after treatment with 0.1 mM TCEP for 10 min at room temperature. ATPase activity of unlabelled Pgp treated with TCEP under the same conditions was used as a control. Data points represent the mean ± range for duplicate measurements.
measuring bimane absorption and fluorescence properties in both free and bound states. Several reports that used bimane labelling of protein constructs containing a single Cys residue showed that the stoichiometry of labelling can be easily calculated as the molar ratio of label to protein, both of which were measured spectroscopically (76,79). However, the presence of additional Cys residues on the target protein will compromise the measurement, due to the different properties of labelled Cys residues located in different environments. Thus the novel bimane derivative, PDT-bimane, which covalently labels target proteins with reversible bonds, was developed to overcome the problem. Cleavage of the bimane label by reducing agents (see section 3.5.1) results in release of the fluorescent group, which can be quantified accurately in its free form. PDT-bimane was characterized as a labelling reagent, and the methodology for determining the stoichiometry of protein labelling using fluorescence spectroscopy was demonstrated by Mansoor and Farrens (77).

3.5.1 Bimane cleavage by TCEP

One of the most important features of using PDT-bimane to label the target protein is that instead of creating a thioether bond, which is irreversible, it forms a disulfide bond which can be cleaved by a reducing agent. TCEP was selected because it has several advantages over other reducing agents, including high stability at pH 7.2, rapid reduction at pH 8 and below, and effectiveness over a wide pH range (1.5 – 8) (92). The total amount of PDT-bimane (covalently bound+free) and the amount of free PDT-bimane in the labelled Pgp sample, were both determined by a protocol combining TCEP
reduction and TCA precipitation (see Figure 3.7) (77). Since most of the previous studies using PDT-bimane were performed using soluble proteins, it was necessary to define the optimum conditions under which TCEP could completely break down the covalent bond between Pgp and bimane. Mansoor et al. (77) used a 5- to 10-fold molar excess of TCEP to remove the pyridyl group from the bimane moiety. However, the concentration required to complete the reaction may vary in different buffer systems (92). In addition, it was reported that reduced PDT-bimane fluorescence intensity can be enhanced at pH 4, so all the samples were mixed with TCA to make a final concentration of 6% prior to fluorescence measurement. This mimics the condition used in protein precipitation, which also minimizes the difference in bimane fluorescence intensity at different pH values.

The TCEP concentration dependence of free PDT-bimane reduction was determined by measuring the increase in bimane fluorescence resulting from cleavage of the disulfide bond. Results showed that after 30 min incubation, the minimum concentration of TCEP required to maximize the cleavage of 10 µM PDT-bimane is 0.5 mM (Figure 3.8). This TCEP concentration was used for further optimization experiments. The time dependence of TCEP reduction of free PDT-bimane was determined by measuring the increase in bimane fluorescence at different incubation times with 0.5 mM TCEP. The TCEP reduction of free PDT-bimane showed the maximum reduction within 10 min (Figure 3.9). The maximum reduction of labelled Pgp with 0.5 mM TCEP was reached within 20 min (Figure 3.10).
Figure 3.7 Stoichiometry determination of bimane labelling

\[
\text{(Bound bimane)} = \text{(Total bimane)} - \text{(Free bimane)}
\]
Figure 3.8  Concentration dependence of TCEP reduction of free PDT-bimane

Free PDT-bimane (2 µM) was incubated with various concentrations of TCEP (0 – 1000 µM) for 30 min at room temperature in the presence of 6% (w/v) TCA. Data points represent the mean ± range for duplicate measurements. Where error bars are not visible, they are contained within the symbols.
Figure 3.9 TCEP reduction of free PDT-bimane

Free PDT-bimane (2 µM) was treated with 0.5 mM TCEP in the presence of 6% (w/v) TCA for various incubation times (0 – 30 min) at room temperature, and bimane fluorescence was measured. Data points represent the mean ± range for duplicate measurements.
Figure 3.10 TCEP reduction of Pgp-bimane(-ADP)

Bimane-labelled Pgp (~10 µg) was treated with 0.5 mM TCEP for various incubation times (0 – 30 min) at room temperature and TCA was added to make a final concentration of 6% (w/v) prior to bimane fluorescence measurement. Data points represent the mean ± range for duplicate measurements.
3.5.2 pH dependence of bimane fluorescence

It has been reported that free reduced PDT-bimane fluorescence intensity is pH dependent, whereas that of PDT-bimane attached to protein is not (77). This was confirmed in the present study. It was suggested that this effect arises from the protonation of the sulfhydryl group at lower pH values. This property can be applied for detecting trace amounts of PDT-bimane. The two steps required prior to fluorescence measurement are TCEP cleavage of Pgp-bimane and pH adjustment of the sample to an acidic range. The fluorescence intensity of reduced PDT-bimane is enhanced by the lower pH of the solution; thus, a trace amount of PDT-bimane can be quantified more accurately. Since reduced PDT-bimane is obtained from free PDT-bimane in the presence of TCA, the effect of TCA concentration on reduced PDT-bimane fluorescence is demonstrated in Figure 3.11. The maximum fluorescence intensity of reduced PDT-bimane is reached at 1% (w/v) TCA, and its fluorescence intensity remains at the same level at higher TCA concentrations. This result indicates that protein precipitation with TCA, which uses higher concentrations of TCA, will not affect the fluorescence intensity of reduced PDT-bimane.

3.5.3 Bound PDT-bimane estimation

By following the method of Mansoor and Farrens (77), bound bimane was estimated by subtracting the measured free PDT-bimane from the total amount of bimane in the Pgp sample. A standard curve was generated by reducing various concentrations of PDT-bimane in the range 0 – 10 µM with 0.1 mM TCEP and
Figure 3.11  Effect of TCA concentration on reduced PDT-bimane fluorescence intensity

The fluorescence emission intensity of 1 µM reduced PDT-bimane was measured at room temperature in the presence of increasing concentrations of TCA. Data points represent the mean ± range for duplicate measurements.
measuring the bimane fluorescence intensity of the samples and standards (Figure 3.12A). The fluorescence intensities of both free PDT-bimane and total PDT-bimane samples were converted into µM units using the standard curves. To calculate the stoichiometry of Pgp labelling, the protein molar concentration obtained from Lowry’s assay and bound bimane molar concentration were used in the following equation:

\[
\text{Stoichiometry of labelling} = \frac{\text{Molar concentration of bound PDT} - \text{bimane}}{\text{Molar concentration of Pgp}}
\]

In this study, both Pgp-bimane(-ADP) and Pgp-bimane(+ADP) were used for stoichiometry determination, as well as unlabelled Pgp as a control. A flow chart illustrating the processes of stoichiometry determination is shown in Figure 3.7. Results showed that the stoichiometry of labelling of Pgp-bimane(-ADP) and Pgp-bimane(+ADP) is 6.9 ± 0.8 and 4.8 ± 0.5, respectively (n = 4). The low amounts of free PDT-bimane in the Pgp-bimane samples, ~10% of total PDT-bimane, suggested good separation efficiency of excess PDT-bimane from labelled Pgp by gel filtration. The difference in the stoichiometry of labelling between Pgp-bimane(-ADP) and Pgp-bimane(+ADP) is approximately 2, which implies that ADP blocks two Cys residues from PDT-bimane labelling. To validate the stoichiometry of Pgp labelling, a sample of a purified single Cys residue protein, colicin E1 S354C (provided by the Merrill laboratory), was labelled with PDT-bimane using the same method. Results showed that the stoichiometry of labelling of colicin E1 S354C was 1.0 ± 0.1 (n = 3), as expected.
Figure 3.12 Standard curve for estimating PDT-bimane concentration and stoichiometry determination

A) Standard curve of PDT-bimane (0 – 10 µM) was performed in the presence of 6% (w/v) TCA at room temperature. Data points represent the mean ± range for duplicate measurements; the error bars fall within the symbols where they are not visible.

B) Free PDT-bimane and total PDT-bimane samples were prepared for Pgp-bimane(-ADP), Pgp-bimane(+ADP), and colicin E1 S354C as described in section 2.6.3, followed by bimane fluorescence measurements. Fluorescence intensities were normalized for protein concentration. Data points represent the mean ± range for duplicate measurements.
3.6 Double labelling of Pgp with PDT-bimane and MIANS

PDT-bimane labelling with Pgp in the presence of ADP was expected to protect the two Cys residues located in the NBDs, so that the protein would still function to bind and hydrolyze ATP. Our lab previously studied Pgp labelling on these two Cys residues with the fluorescent probe MIANS (26). Using the fluorescence properties of MIANS, which shows very low fluorescence emission in the free state, but high intensity in the conjugated state, the reaction of Pgp with MIANS can be monitored in real time. The ability of MIANS to react with two Cys residues in Pgp was previously confirmed by the stoichiometry of labelling (26). The blocking of MIANS labelling by ATP was also shown to be concentration dependent, suggesting that the two labelled Cys residues were located in the NBDs. The fluorescence spectra of free MIANS, compared to MIANS reacted with DTE, and to Pgp conjugated with MIANS, are shown in Figure 3.13. Free MIANS showed no fluorescence emission, but fluorescence emission appeared in the DTE-reacted form, with an emission peak at 443 nm. In addition, a blue shift of 28 nm is observed for MIANS reacted with Pgp, compared to MIANS reacted with DTE. This effect was previously noted (26), and indicates that MIANS is bound in a hydrophobic region of the NBDs of Pgp.

Based on the evidence in our previous study, the two Pgp adducts, Pgp-bimane(+ADP) and Pgp-bimane(-ADP), were treated with MIANS to determine whether there are available Cys residues for covalent reaction. The Pgp-bimane(+ADP) adduct, which still retained ATPase activity, was treated with MIANS to initiate the labelling reaction, and the MIANS fluorescence intensity
was monitored in real time. As shown in Figure 3.14, as anticipated, Pgp-bimane(+ADP) showed a rapid increase in fluorescence intensity, indicating reaction with MIANS. Adding ADP prior to MIANS prevented the reaction, suggesting that the two Cys residues available for labelling with MIANS are in the NBDs. In contrast, Pgp-bimane(-ADP) showed no fluorescence increase following addition of MIANS, similar to the negative control. This observation strongly suggests that there are no available Cys residues on Pgp-bimane(-ADP), indicating that all of them have been labelled with bimane.

3.7 Fluorescence properties of Pgp-bimane

The fluorescence emission spectra of Pgp-bimane(-ADP) and Pgp-bimane(+ADP) (Figure 3.15) show the peak of emission at the same wavelength of 467 nm. Both Pgp-bimane adducts display a blue shift of 18 nm when compared to the emission spectrum of PDT-bimane reduced with TCEP. This blue shift indicates that the bimane groups are located in a less polar environment when covalently linked to Pgp. To confirm the bimane labelling of Pgp, Pgp-bimane was run on SDS-PAGE, followed by fluorescence imaging. Both Pgp-bimane(-ADP) and Pgp-bimane(+ADP) showed a band at 170 kDa on fluorescence imaging and Coomassie blue stain (Figure 3.16) while unlabelled Pgp can be visualized only using Coomassie blue stain.

3.8 CD spectroscopy

The CD measurements were performed by Peihua Lu. Figure 3.17 shows that the melting points ($T_m$) of Pgp-bimane(-ADP) and Pgp-bimane(+ADP) in 2
mM CHAPS/20 mM HEPES buffer are 53.5 and 55.2°C, respectively, suggesting that they are still relatively well-folded compared to unlabelled Pgp ($T_m = 54.7^\circ$C). Thus the labelling process does not disrupt the secondary structure of Pgp.

3.9 Discussion

Previous studies in our laboratory used bimane derivatives, including monobromobimane and dibromobimane, as fluorescent probes to label Pgp. These had the disadvantage of forming irreversible covalent bonds that complicated the quantitation of labelling on any protein containing more than one Cys residue. Covalent labelling by PDT-bimane can overcome this problem because the reversible disulfide bond can be broken by reducing agents. With this fluorescent probe, bound bimane can be quantitated without any complication from multiple Cys residues, and the sensitivity of bimane measurement was improved. The goal of this project was to covalently bind PDT-bimane to Cys residues within (or close to) the substrate-binding pocket of Pgp. To ensure that after bimane labelling Pgp was still able to function as a transporter, the labelling step was thoroughly characterized. By adding ADP to purified Pgp prior to labelling, the ATPase activity of labelled Pgp was retained at a relatively high level (53%) compared to unlabelled Pgp. In contrast, Pgp labelled in the absence of ADP lost all its activity. By treating Pgp-bimane(-ADP) and Pgp-bimane(+ADP) with TCEP, the covalent bond between Pgp and bimane was cleaved, and the ATPase activity of labelled Pgp (Pgp-bimane(-ADP) and Pgp-bimane(+ADP)) recovered to ~90%, suggesting that the folding and conformation of Pgp still remained intact after bimane labelling. CD denaturation
Figure 3.13  Fluorescence spectrum of free MIANS and MIANS conjugated to DTE and Pgp

MIANS fluorescence emission spectra were collected at 22°C for free MIANS (4 µM), MIANS reacted with DTE (4 µM) and MIANS conjugated to Pgp (50 µg/mL), all in 2 mM CHAPS/20 mM HEPES buffer.
Figure 3.14 MIANS labelling of Pgp-bimane adducts

Pgp-bimane(-ADP) and Pgp-bimane(+ADP) (50 µg/mL in 2 mM CHAPS/HEPES buffer) were mixed with 4 µM MIANS at 22°C, and the MIANS fluorescence intensity was monitored in real time. Identical experiments were carried in the presence of 5 mM ADP to block MIANS reaction at the Cys residues in the NBDs. 4 µM MIANS in 2 mM CHAPS/20 mM HEPES buffer was used as a control for correction of the spectra.
**Figure 3.15  Fluorescence spectra of reduced PDT-bimane and Pgp-bimane adducts**

Fluorescence emission spectra were recorded for PDT-bimane (2 µM) following treatment with 0.1 mM TCEP, and Pgp-bimane adducts (100 µg/mL), in 2 mM CHAPS/20 mM HEPES buffer at 22°C. Reduced PDT-bimane shows a peak at 485 nm, while both emission spectra of bimane-labelled Pgp (Pgp-bimane(-ADP) and Pgp-bimane(+ADP)) show a peak at 467 nm.
Figure 3.16  SDS-polyacrylamide gel electrophoresis analysis of bimane-labelled Pgp

Pgp-bimane(-ADP) (2.5 µg) was run on a 10% polyacrylamide gel. Samples were visualized by (A) fluorescence emission imaging ($\lambda_{ex} = 302$ nm, 595 nm cut-on emission filter), followed by (B) Coomassie blue staining prior to white reflective imaging. The bimane-labelled Pgp band corresponds to a molecular mass of 170 kDa.
Figure 3.17  Melting curves of Pgp and Pgp-bimane adducts as determined by CD spectroscopy

The CD spectra of unlabelled Pgp and Pgp-bimane adducts in 2 mM CHAPS/20 mM HEPES buffer were measured along an increasing temperature gradient from 30 - 70°C. Plots of the ellipticity at 222 nm (reflecting α-helical content) for Pgp (long-dashed line), Pgp-bimane(+ADP) (short-dashed line), and Pgp-bimane(-ADP) (solid line) were normalized and fitted using SigmaPlot. The melting temperatures of Pgp, Pgp-bimane(+ADP), and Pgp-bimane(-ADP) are 54.7°C, 55.2°C, and 53.5°C, respectively.
experiments indicated that both labelled Pgp-bimane adducts were correctly folded and of similar stability to unlabelled Pgp.

Since hamster Pgp contains 7 Cys residues, optimization was required to ensure that the labelling reaction was complete. The goal was to ensure consistency of labelling and to define the conditions that reached the maximum level of labelling. Pgp ATPase activity was used as a parameter to monitor the labelling reaction. Interestingly, PDT-bimane was able to completely inhibit the ATPase activity of Pgp (similar to NEM) while the other bimane derivatives inhibited activity by only ~40 – 50%. Previous studies suggested using 10- to 15-fold PDT-bimane molar concentration relative to protein during the labelling reaction; however, these were performed on single Cys proteins which might not be suitable in our case (77,93). The concentration dependence of PDT-bimane on Pgp ATPase activity showed that 0.1 mM PDT-bimane can completely inhibit ATPase activity, with labelling completed in less than 10 min. The conditions selected for routine labelling were 0.5 mM PDT-bimane and 30 min incubation time at 37°C for Pgp-bimane(-ADP), with additional incubation with 5 mM ADP prior to labelling for Pgp-bimane(+ADP). Bimane-labelled Pgp was efficiently separated from free PDT-bimane by gel filtration chromatography. A protocol was developed for measuring free and total PDT-bimane, involving TCEP cleavage of bimane, followed by fluorescence enhancement under acidic conditions. By measuring the free and total amount of PDT-bimane separately, PDT-bimane covalently bound to Pgp was estimated. The obtained stoichiometry of labelling for Pgp-bimane(-ADP) and Pgp-bimane(+ADP) were 7 and 5 respectively. Taken
together with the ability of ADP to protect Pgp from loss of catalytic activity, these results indicate that ADP prevents covalent labelling of two Cys residues, likely those in the Walker A motifs of the NBDs.

Double labelling with MIANS, which was reported previously to specifically label only the two Cys residues in the NBDs, showed that those Cys are still available in Pgp-bimane(+ADP), and can be protected from MIANS labelling by ADP. Thus, ADP protects the two Cys residues located in the NBDs from bimane labelling. As a result, we can prepare bimane-labelled Pgp with good ATPase activity, which is anticipated to retain efflux transport function. Both bimane-labelled Pgp adducts showed similar fluorescence emission spectra. An 18 nm blue shift compared to free reduced PDT-bimane indicated that the labelled Cys residues are located in a relatively nonpolar local environment.
Chapter 4

Drug binding properties of Pgp-bimane
4.1 Introduction

Several mutagenesis studies have shown that particular regions within the TM helices of both halves of Pgp are involved in binding of substrates. This agrees with evidence obtained from photoaffinity labelling using photo-reactive substrates, including $^3$H-azidopine (94,95), $^{125}$I-iodoaryl-azidoprazosin (96) and propafenone (97), which showed that the labelled regions are located in both halves of Pgp. Crystal structures of Pgp from mouse (28) and C. elegans (29) have shown the location and orientation of the helices spanning the membrane. However, very few crystal structures of eukaryotic ABC exporters, including Pgp from mouse (28) and Atm1 from Novosphingobium aromaticivorans (98), show a substrate bound inside the binding pocket.

The existence of H- and R-sites in the substrate-binding pocket has been suggested, where the H- and R-sites preferentially bind and transport H33342 and rhodamine 123, respectively (45). The binding pocket of Pgp is large (28) and is known to be able to accommodate two substrates simultaneously (28,46,50), as well as large covalent drug dimers (99). Many studies have investigated and attempted to define the substrate-binding sites of Pgp, yet none of these can completely specify their location. Fluorescence spectroscopy is a useful complementary approach to determine the binding of substrates, which causes a conformational change of Pgp, thus affecting intrinsic or covalently-bound fluorophores (9,26).
The goal of this chapter was to determine the dissociation constants for binding of three substrates to native Pgp and bimane-labelled Pgp. Both Trp quenching by TMR, LDS-751 and H33342, and bimane quenching by TMR and LDS-751, were employed to estimate \( K_d \) values. A comparison between the \( K_d \) value for substrate binding to native Pgp and bimane-labelled Pgp was anticipated to reflect the effect of bimane, covalently linked within or close to the binding pocket, and on the ability of Pgp to bind a second substrate. Results might provide information on the size and multi-site nature of the binding pocket.

4.2 Drug binding affinity of Pgp-bimane using Trp fluorescence

The binding affinity of three substrates, TMR, H33342 and LDS-751, to bimane-labelled Pgp (Pgp-bimane(-ADP) and Pgp-bimane(+ADP)) was determined by intrinsic Trp fluorescence quenching. Results showed concentration-dependent saturable quenching of Trp fluorescence (\( \lambda_{ex}/\lambda_{em} = 290/330 \text{ nm} \)) which was fitted to a binding equation, and \( K_d \) and \( \Delta F_{\text{max}} \) values were extracted (Figures 4.1 – 4.3). A parallel experiment with the three substrates was also carried out with unlabelled Pgp (top panels in Figures 4.1 – 4.3). The estimated values of \( K_d \) and \( \Delta F_{\text{max}} \) for all three substrates with Pgp, Pgp-bimane(-ADP) and Pgp-bimane(+ADP) are shown in Table 4.1. The binding to unlabelled Pgp of these substrates has been characterized previously (46,47), and showed similar values to those obtained here. In general, unlabelled and bimane-labelled Pgp showed similar binding affinity for all three substrates (within a 3-fold range). These results suggest that the substrate-binding pocket of
Figure 4.1  Binding of TMR to unlabelled Pgp and Pgp-bimane adducts determined using Trp fluorescence quenching

Unlabelled Pgp and Pgp-bimane adducts (50 µg/mL in 2 mM CHAPS/20 mM HEPES buffer) were titrated at 22°C with TMR and the % Trp fluorescence quenching was determined after correction (see section 2.7.3). Data points represent the mean ± range for duplicate determinations. The data were fitted to a binding equation (section 2.7.3), indicated by the solid line.
Figure 4.2  Binding of LDS-751 to unlabelled Pgp and Pgp-bimane adducts determined using Trp fluorescence quenching

Unlabelled Pgp and Pgp-bimane adducts (50 µg/mL in 2 mM CHAPS/20 mM HEPES buffer) were titrated at 22°C with LDS-751 and the % Trp fluorescence quenching was determined after correction (see section 2.7.3). Data points represent the mean ± range for duplicate determinations. The data were fitted to a binding equation (section 2.7.3), indicated by the solid line.
Figure 4.3  Binding of H33342 to unlabelled Pgp and Pgp-bimane adducts determined using Trp fluorescence quenching

Unlabelled Pgp and Pgp-bimane adducts (50 µg/mL in 2 mM CHAPS/20 mM HEPES buffer) were titrated at 22°C with H33342 and the % Trp fluorescence quenching was determined after correction (see section 2.7.3). Data points represent the mean ± range for duplicate determinations. The data were fitted to a binding equation (section 2.7.3), indicated by the solid line.
Pgp with 3 bimane groups within or close to the cavity is large enough to accommodate bimane and another drug simultaneously. In addition, even though Pgp-bimane(-ADP) showed no ATPase activity, it was able to bind substrates with similar affinity to that of Pgp-bimane(+ADP), which retains substantial ATPase activity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pgp</th>
<th>Pgp-bimane(-ADP)</th>
<th>Pgp-bimane(+ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (µM)</td>
<td>$\Delta F_{max}$ (%)</td>
<td>$K_d$ (µM)</td>
</tr>
<tr>
<td>TMR</td>
<td>1.2 ± 0.3</td>
<td>15.9 ± 1.4</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>LDS-751</td>
<td>1.7 ± 0.8</td>
<td>12 ± 2.1</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>H33342</td>
<td>1.7 ± 1.0</td>
<td>21.8 ± 4.3</td>
<td>1.4 ± 0.6</td>
</tr>
</tbody>
</table>

$K_d$ and $\Delta F_{max}$ were estimated from the Trp quenching curves shown in Figures 4.1 – 4.3. Errors represents the goodness of fit to the binding equation (section 2.7.3).

4.3 Drug binding affinity of Pgp-bimane using bimane fluorescence

It was anticipated that bimane group distributed within Pgp could be used as an extrinsic fluorescence probe in a similar way to Trp fluorescence. This not only ensures the accuracy of $K_d$ values determined by Trp fluorescence quenching, but also demonstrates an alternative fluorescence probe that can be used for $K_d$ determination. How the substrates TMR and LDS-751 bind to Pgp-bimane was further characterized using bimane fluorescence. H33342 could not
be used, due to overlap of its fluorescence emission with that of bimane. The bimane fluorescence ($\lambda_{ex}/\lambda_{em} = 381/478$ nm) quenching data for TMR and LDS-751 were plotted against substrate concentration, and fitted to a binding equation (Figure 4.4). The $K_d$ and $\Delta F_{\text{max}}$ values for binding of TMR and LDS-751 to bimane-labelled Pgp are shown in Table 4.2. The binding affinities of TMR and LDS-751 to Pgp-bimane(-ADP) and Pgp-bimane(+ADP) determined by bimane fluorescence quenching are very similar, and comparable to the values determined by Trp fluorescence quenching (Table 4.1). Interestingly, the $\Delta F_{\text{max}}$ values for both TMR and LDS-751 determined by bimane quenching, 54% and 80%, respectively, are substantially higher than those obtained by Trp quenching (10 – 16%).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pgp-bimane(-ADP)</th>
<th>Pgp-bimane(+ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (µM)</td>
<td>$\Delta F_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>TMR</td>
<td>1.9 ± 0.2</td>
<td>57 ± 2.6</td>
</tr>
<tr>
<td>LDS-751</td>
<td>2.1 ± 0.4</td>
<td>82 ± 5.1</td>
</tr>
</tbody>
</table>

$K_d$ and $\Delta F_{\text{max}}$ were estimated from the bimane quenching curves shown in Figure 4.4. Errors represents the goodness of fit to the binding equation.

4.4 Discussion

To date, the only extrinsic fluorescence probe employed to label Pgp is MIANS, which covalently reacts in the NBDs and results in complete loss of
Figure 4.4  Binding of TMR to Pgp-bimane adducts determined using bimane fluorescence quenching

Pgp-bimane adducts (50 µg/mL in 2 mM CHAPS/20 mM HEPES buffer) were titrated at 22°C with (A) TMR or (B) LDS-751, and the % bimane quenching was determined after correction (see section 2.7.3). Data points represent the mean ± range for duplicate determinations. The data were fitted to a binding equation (section 2.7.3), indicated by the solid line for Pgp-bimane(-ADP) and the dashed line for Pgp-bimane(+ADP). Where error bars are not visible, they are contained within the symbols.
ATPase activity (26). In this study, bimane fluorescence quenching was used to
determine the binding affinity of substrates for the first time. One advantage of
using bimane is that it provides an alternative wavelength for measurements
involving fluorescent drugs whose spectra might overlap with Trp or MIANS
fluorescence. A second advantage is that the labelled Pgp is still catalytically
active for Pgp-bimane(+ADP). From these results, bimane is shown to be a good
extrinsic fluorescent probe, which provides an alternative to MIANS for $K_d$
determination.

It has been proposed that Pgp can bind and possibly transport two or
more drugs simultaneously; however, the evidence supporting this idea is limited.
In this study, the binding affinities of three fluorescent substrates for bimane-
labelled Pgp showed no significant differences from native Pgp. This observation
implies that labelled Pgp can bind the second drug when bimane occupies part of
the substrate-binding pocket. It is possible that bimane occupies the binding
pocket in a sub-site that does not overlap with all three substrates, which
includes compounds that occupy both the H- and the R-sites. The relatively small
size of the bimane molecule might not be sufficient to perturb the binding sub-
sites of the three substrates. Also, the flexibility of the substrate-binding pocket
may allow TM helices to shift to fit the second drug without changing the binding
affinity. Another explanation for the unchanged substrate binding affinity is that
the bimane groups are not oriented towards the inside of the substrate-binding
pocket. However, since three bimane groups are present within the TM regions,
and the folding and enzymatic activity of the labelled protein are not impaired, it
seems likely that one or more of them would occupy space in the binding pocket. The relatively high $\Delta F_{\text{max}}$ for bimane quenching by both TMR and LDS-751 compared to that observed for Trp quenching, suggests that either one or more bimane groups are located close to the bound substrate, or efficient FRET contributes to the quenching mechanism.
Chapter 5

Drug transport properties of Pgp-bimane
5.1 Introduction

Pgp has been proposed to be a “vacuum cleaner” for hydrophobic molecules present within the lipid bilayer (100). The mechanism of Pgp-mediated drug transport was suggested to involve two pseudosymmetric translocation pathways inside the binding pocket (70). Site-directed mutagenesis was used to replace an amino residue located in close proximity to one of the putative binding sites with an Arg residue, which was anticipated to interfere with one of the translocation pathways. The transport of rhodamine 123 was partially lost, and one transport site was affected more than the other site. However, if the residues in both binding sites were replaced, the ability of Pgp to transport drugs was completely lost. The relationship between these transport pathways and the binding pocket, and the preferential binding site of substrates, are still not fully understood.

In the present chapter, the first goal was to use transport experiments to determine whether PDT-bimane interacts with the H- or R-site of Pgp. The inhibitory effect of PDT-bimane on transport of H33342 and TMR was used to identify how PDT-bimane competes with an H-site substrate (H33342) and an R-site substrate (TMR).

Loo and Clark showed that two different substrates can fit inside the Pgp substrate-binding pocket (50). This seems to agree with our results of the substrate binding affinity of bimane-labelled Pgp, which showed similar $K_d$ values to unlabelled Pgp (Chapter 4). This leads to the question of whether Pgp with
one substrate occupying the binding pocket still retains transport function. The second goal of this chapter was to address this issue. Transport functions were compared between native Pgp and Pgp-bimane(+ADP), which still retained some ATPase activity.

5.2 Reconstitution of unlabelled Pgp and Pgp-bimane adduct

For drug transport studies, Pgp was reconstituted into egg PC proteoliposomes by gel filtration chromatography as described in section 2.8.1. A mixture of Pgp and egg PC in CHAPS was loaded on to a Sephadex G-50 column. Proteoliposomes emerged at the void volume of the column. A Bradford protein assay and an ATPase assay were used to follow the proteoliposomes that eluted from the column (Figure 5.1). The fractions with protein concentration > 0.2 mg/mL were pooled and used for transport assays.

5.3 PDT-bimane inhibition of drug transport by unlabelled Pgp

Proteoliposomes of unlabelled Pgp were used to determine whether free PDT-bimane is a substrate for the H-site, R-site, or both. TMR and H33342, which represent the H- and R-site substrates, respectively, were used as substrates for transport, and the inhibitory effect of free PDT-bimane on transport of each substrate was determined. The inhibitory/stimulatory effect of free PDT-bimane on Pgp ATPase activity cannot be measured, due to the fast reaction of PDT-bimane compared to the 30-min duration of the ATPase assay. However, the transport assay is very fast with data collected in < 30s after initiation of transport by addition of ATP (see Figure 5.2). With this strategy, the
Figure 5.1  Reconstitution of Pgp into egg PC proteoliposomes by gel filtration chromatography

Pgp was reconstituted into proteoliposomes of egg PC by gel filtration chromatography on a Sephadex G-50 column at 4°C to remove CHAPS from the Pgp-egg PC-CHAPS mixture. Individual fractions of 0.5 mL were collected, followed by Bradford protein assay (A_{595}) and ATPase assay (A_{750}) to monitor the reconstitution. Proteoliposomes elute at the void volume of the column.
Figure 5.2  Estimation of initial rate of TMR transport

(A) TMR transport was monitored by measuring the decrease in fluorescence intensity after adding ATP to Pgp proteoliposomes in the presence of 1 µM TMR. The initial rate of drug transport was estimated by fitting the data over the period 10 – 30 s after ATP addition to a linear curve. (B) The data in the selected time frame (black box) are shown in an expanded scale.
effect of the PDT-bimane labelling reaction could be minimized. Transport of TMR and H33342 was carried out by real-time fluorescence measurements (section 2.8.2). The initial rates of drug transport were estimated from the linear region of the data 10 – 30 s following the addition of ATP and PDT-bimane (see Figure 5.2A and B for TMR). When TMR transport was carried out in the presence of increasing concentrations of free PDT-bimane, the initial rate of transport decreased, and was completely abolished at 200 µM (Figure 5.3A). Similar results were obtained for transport of H33342 in the presence of free PDT-bimane, with transport abolished at 100 µM (Figure 5.4A). The initial rates of TMR and H33342 transport were plotted against PDT-bimane concentration, and the IC$_{50}$ values for transport inhibition were extracted using SigmaPlot (Figures 5.3B and 5.4B). The IC$_{50}$ values obtained for TMR and H33342 transport were 20.7 µM and 30.5 µM, respectively, suggesting that the PDT-bimane transport site overlaps with both the H- and R-sites. For PDT-bimane inhibition of H33342 transport, a small amount of overlap between H33342 and bimane fluorescence leads to the upward trend of the fluorescence trace at 100 µM PDT-bimane (Figure 5.4A), thus resulting in underestimation of the IC$_{50}$ value.

5.4 Drug transport by Pgp-bimane

In order to characterize the transport function of the Pgp-bimane(+ADP) adduct, studies were carried out to determine the effect of bimane labelling on the kinetic parameters, K$_M$ and V$_{max}$, for TMR, H33342 and LDS-751 transport. To achieve this, the initial rates of drug transport at different concentrations of the
three substrates were measured and plotted against their concentration (Figures 5.5, 5.6 and 5.7). The values of $K_M$ and $V_{\text{max}}$ for transport of the three substrates by native Pgp and bimane-labelled Pgp are summarized in Table 5.1. Bimane-labelled Pgp retained substantial amounts of transport activity, which obeyed apparent Michaelis-Menten kinetics for all three substrates. The $V_{\text{max}}$ values for Pgp-bimane(+ADP) were reduced by 3- to 5-fold, although $V_{\text{max}}$ for TMR transport was reduced the most. Interestingly, the differences in $K_M$ values between native Pgp and Pgp-bimane(+ADP) were only within ~2-fold.

Table 5.1  The effect of bimane labelling on drug transport by Pgp reconstituted into egg PC proteoliposomes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pgp</th>
<th>Pgp-bimane(+ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR</td>
<td>$K_M$</td>
<td>0.20 µM</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>2.3 %.s$^{-1}$</td>
</tr>
<tr>
<td>H33342</td>
<td>$K_M$</td>
<td>0.22 µM</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>0.84 %.s$^{-1}$</td>
</tr>
<tr>
<td>LDS-751</td>
<td>$K_M$</td>
<td>0.49 µM</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>1.6 %.s$^{-1}$</td>
</tr>
</tbody>
</table>

5.5 Discussion

By using a rapid drug transport assay, the interaction of PDT-bimane with the H- and R-sites of Pgp can be determined with the effect of the labelling reaction minimized. Typically, R-site substrates compete with TMR transport, while H-site substrates compete with H33342 transport. PDT-bimane inhibited
Figure 5.3  PDT-bimane inhibition of TMR transport

Unlabelled Pgp proteoliposomes in 20 mM HEPES buffer were incubated with 1 µM TMR for 10 min on ice in the dark prior to the measurement. (A) The initial rate of drug transport was measured after adding 1 mM ATP to the proteoliposomes at time zero by monitoring TMR fluorescence emission intensity. The initial rate of TMR transport at various concentrations of free PDT-bimane was calculated from the slope in the time frame 10 – 30 s after addition of ATP. (B) The initial rate of TMR transport was plotted against PDT-bimane concentrations, fitted to a hyperbolic curve, and the IC$_{50}$ value was extracted. Data points represent the mean ± range for duplicate determinations.

IC$_{50}$ = 20.7 µM
Figure 5.4  PDT-bimane inhibition of H33342 transport

Unlabelled Pgp proteoliposomes in 20 mM HEPES buffer were incubated with 1 µM H33342 for 10 min on ice in the dark prior to the measurement. (A) The initial rate of drug transport was measured after adding 1 mM ATP to the proteoliposomes at time zero by monitoring H33342 fluorescence emission intensity. The initial rate of H33342 transport at various concentrations of free PDT-bimane was calculated from the slope in the time frame 10 – 30 s after addition of ATP. (B) The initial rate of H33342 transport was plotted against PDT-bimane concentrations, fitted to a hyperbolic curve, and the IC₅₀ value was extracted. Data points represent the mean ± range for duplicate determinations.
Figure 5.5  TMR transport by Pgp and Pgp-bimane(+ADP) reconstituted into egg PC proteoliposomes

Unlabelled Pgp proteoliposomes and Pgp-bimane(+ADP) proteoliposomes in 20 mM HEPES buffer were incubated with TMR for 10 min on ice in the dark prior to the measurement. Transport was started by addition of ATP, and the initial rate of drug transport was determined. Data points were fitted to the Michaelis-Menten equation. Data points represent the mean ± range for duplicate determinations. Where error bars are not visible, they are contained within the symbols.
Figure 5.6  H33342 transport by Pgp and Pgp-bimane(+ADP) reconstituted into egg PC proteoliposomes

Unlabelled Pgp proteoliposomes and Pgp-bimane(+ADP) proteoliposomes in 20 mM HEPES buffer were incubated with H33342 for 10 min on ice in the dark prior to the measurement. Transport was started by addition of ATP, and the initial rate of drug transport was determined. Data points were fitted to the Michaelis-Menten equation. Data points represent the mean ± range for duplicate determinations. Where error bars are not visible, they are contained within the symbols.
Figure 5.7  LDS-751 transport by Pgp and Pgp-bimane(+ADP) reconstituted into egg PC proteoliposomes

Unlabelled Pgp proteoliposomes and Pgp-bimane(+ADP) proteoliposomes in 20 mM HEPES buffer were incubated with LDS-751 for 10 min on ice in the dark prior to the measurement. Transport was started by addition of ATP, and the initial rate of drug transport was determined. Data points were fitted to the Michaelis-Menten equation. Data points represent the mean ± range for duplicate determinations. Where error bars are not visible, they are contained within the symbols.
the transport of both TMR and H33342 with similar IC$_{50}$ values. This could be explained by the binding site of PDT-bimane overlapping both the TMR binding and H33342 sites, or PDT-bimane could compete with both sites simultaneously. In other words, two PDT-bimananes could occupy the pocket to block both H- and R-sites.

Another goal of this chapter is to determine whether covalent binding of PDT-bimane to Cys residues in the binding pocket would be sufficient to completely inhibit the transport function of Pgp. Hamster Pgp contains 7 Cys residues, two of which are located in the conserved Walker A motif, which are responsible for the complete loss of ATPase activity after NEM and MIANS labelling (26,27). Pgp-bimane(+ADP) retained ~53% ATPase activity, and can be used in transport assays, which require ATP hydrolysis to energize the transport cycle. According to the results presented in this chapter, the Pgp-bimane(+ADP) adduct clearly still binds and transports all three substrates across the membrane, although with a decreased rate compared to native Pgp. The substantial loss of drug transport ability by Pgp-bimane may arise in two ways: a partial loss of ATPase activity, which is essential to energize the transport cycle, and effects of covalently-linked bimane on the TMDs. However, it is still unclear which bimane groups make the major contribution to the loss of drug transport function. It is possible that covalent linkage of bimane to one of the Cys residues in the TMDs (residues 134, 714, and 953) triggered the inhibitory effect on drug transport. Presumably, any or all of these three bimane groups could face the inside of the binding pocket, suggesting that the Pgp-bimane adduct can
accommodate one or more bimane groups in the pocket, and still bind and transport another substrate in one cycle of transport. This also agrees with several studies that used thiol-reactive crosslinking (50), fluorescent titration (49), crystal structure (28), and molecular docking (48), to show that the binding pocket of Pgp can accommodate at least two different substrates at the same time. The results presented here are the first evidence that one of two simultaneously-bound substrates can be transported by Pgp.
Chapter 6

Summary and conclusions
6.1 Summary and conclusions

To date, many studies attempting to define the drug-binding site(s) of Pgp found that the binding pocket can accommodate more than one substrate (28,48-50,101). However, data about whether Pgp can transport molecules when two drugs occupy the binding pocket at the same time are scarce. The work presented in this thesis attempts to provide further evidence that Pgp possibly binds and transports more than one substrate simultaneously.

The objective in Chapter 3 was to prepare a covalently-linked Pgp-drug adduct which retained ATPase activity, with the expectation that it might still function as a transporter. A previous study by our group successfully used a thiol-reactive fluorescent probe, MIANS, to label two Cys residues in the NBDs of hamster Pgp (26). This reaction can be prevented by the occupancy of the nucleotide-binding sites by ATP. MIANS was used as an extrinsic probe to measure the binding affinity of many substrates and nucleotide derivatives. However, the Pgp-MIANS adduct failed to retain ATPase activity, so it could not be used further for drug transport experiments. In this thesis, I used a novel fluorescent probe, PDT-bimane, combined with the addition of ADP to prevent reaction with Cys residues in the NBDs. This resulted in a Pgp-bimane adduct which partially retained ATPase activity. Studies by Loo and Clark (54,56) identified the bimane derivative dibromobimane as a Pgp substrate and used it, combined with Cys-scanning mutagenesis, to identify the key residues in the TMDs essential for drug binding. However, we have shown in Chapter 3 the advantages of using PDT-bimane over other bimane derivatives. The most
important advantage of PDT-bimane is that instead of forming a thioether bond with Cys residues, which is not reversible, it forms a disulfide bond that can be broken down by reducing agents. Since Pgp contains more than one Cys residue, which compromises the measurement of the number of residues being labelled, a breakable disulfide bond helped advance the stoichiometry determination of PDT-bimane labelling using fluorescence spectroscopy. In addition, the fluorescence intensity of the bimane released from labelled Pgp by oxidation can be enhanced in acidic solution, leading to a higher accuracy of determination of labelling stoichiometry. Within the time frame of labelling in the absence of ADP, PDT-bimane completely abolished ATPase activity, whereas other bimane derivatives only showed ~50% inhibition. This assay can be used to monitor completion of the labelling reaction, as shown by comparison to other thiol-reactive agents (NEM (27) and MIANS (26)) which inhibited ATP hydrolysis by 100%. Stoichiometry measurements showed that all 7 Cys residues of Pgp (4 in the NBDs and cytoplasmic region, 3 in the TMDs) were labelled with bimane under these conditions. Labelling of Pgp with PDT-bimane in the presence of ADP resulted in modification of 5 Cys residues with retention of substantial ATPase activity. Double labelling with MIANS showed that the two Cys residues in the NBDs were not labelled under these conditions.

The secondary structure and conformation of native and bimane-labelled Pgp were examined by CD spectroscopy. All three proteins (native Pgp, Pgp-bimane(-ADP), and Pgp-bimane(+ADP)) exhibited similar melting points, suggesting that bimane labelling did not impair the secondary structure or folding
of Pgp. In addition, treating bimane-labelled Pgp with TCEP can restore ATPase activity, suggesting that the conformation of Pgp was still intact after PDT-bimane labelling.

The polyspecific nature of the substrate-binding pocket of Pgp was previously examined by $K_d$ determination using MIANS and Trp fluorescence quenching (9,26). These results suggested that substrates can be discriminated by Pgp, with $K_d$ values spanning a 3000-fold range from 40 nm to 150 µM (42). Another study by our group determined the $K_d$ values for simultaneous binding of two substrates by performing a double titration with LDS-751 and rhodamine 123; however, several corrections are required due to the effect of multiple reversible drug equilibria. Therefore, $K_d$ determination of binding of a second drug to the Pgp-bimane adduct could avoid those complications by having one substrate covalently bound in the binding pocket. Apparently, all three drugs used in the experiments in Chapter 4 showed only small differences in the $K_d$ of binding between native Pgp and bimane-labelled Pgp (less than 3-fold). This result suggests that the three bimane groups linked to Cys residues surrounding the binding pocket have little effect on the binding of another drug molecule to Pgp.

In this thesis, the $K_d$ values for drug binding to the Pgp-bimane adduct were determined using bimane fluorescence for the first time. Several advantages of using bimane fluorescence were found; it provides an alternative wavelength for analysis that may not overlap with that of the substrate of interest, and bimane gives a higher fluorescence quenching on substrate binding. The results in Chapter 4 showed that the $K_d$ values for TMR and H33342 binding to
Pgp-bimane(-ADP) and Pgp-bimane(+ADP) are very similar, suggesting that both labelled Pgps can bind another substrate. Also, $K_d$ values determined by Trp fluorescence and bimane fluorescence are very similar, but bimane displays a higher $\Delta F_{\text{max}}$ value.

In order to locate the three labelled Cys residues in the TMDs of hamster Pgp, the protein sequence of hamster Pgp was aligned with that of mouse Pgp (PDB: 3G5U; (28)) and C. elegans Pgp (PDB: 4F4C; (29)), whose crystal structures have been solved. BLAST was used to define the amino acid residues corresponding to those three Cys residues, which are expected to be positioned close to the substrate-binding pocket. In mouse Pgp, the three Cys residues were assigned to TM2 (C133), TM7 (C713) and TM11 (C952) (Figure 6.1). In C. elegans Pgp, only one of the three residues was conserved as a Cys (C996 in TM11). The Cys in TM2 was replaced by a Leu residue (L161) and the Cys in TM7 was replaced by a Thr residue (T760) (Figure 6.2). However, the location of the three residues relative to the bilayer normal is in the same order between the two structures, TM2, TM7 and TM11, respectively, from the cytoplasmic to the extracellular side. Recently, a study by Ferreira et al. has determined the location of the substrate-binding sites using molecular docking (48). Several substrates were docked into the binding pocket, and the H- and R-sites were positioned close to the cytoplasmic interface. These results seem to agree with previous studies which attempted to identify the drug binding sites, including Cys scanning mutagenesis (56,58,102), a pharmacophore model (103), and FRET (47). It is possible that the location of the covalently-linked bimane groups does not
overlap, or overlaps very little, with the preferential binding sites of other substrates, thus resulting in no significant change in the $K_d$ for binding for TMR, H33342 and LDS-751. However, transport experiments carried out in Chapter 5 indicated that free PDT-bimane competed with transport of these substrates, suggesting that linked bimane groups would occupy part of the binding sub-site for all three of them. MD simulations of mouse Pgp crystallographic conformations indicated that the TMDs are very flexible (104). In addition, Pgp has been proposed to bind drugs by an induced-fit mechanism, which suggests that re-arrangement of the TMDs takes place to accommodate multiple drug molecules. This rearrangement would optimize interaction of the drug molecules with the binding pocket, resulting in only small changes in binding affinity.

Several studies have demonstrated the ability of Pgp to accommodate two substrates simultaneously; however, there is no evidence to show that Pgp can transport two substrates at the same time. In this thesis, we provided evidence that Pgp can transport one substrate when two substrates occupy the binding pocket. One substrate (bimane) was covalently linked to the substrate-binding pocket of Pgp prior to addition of a second substrate. The kinetic parameters of drug transport for TMR, H33342, and LDS-751, were measured for native Pgp and the Pgp-bimane adduct. The results showed that although $V_{\text{max}}$ values of the Pgp-bimane adduct were reduced, clearly, it can still transport the second substrate.
Figure 6.1 Cys residues in the TMDs of mouse Pgp

The crystal structure of mouse Pgp (PDB 3G5U) is shown in ribbon format, with the N-terminal half TMD1 in yellow and the C-terminal half TMD2 in red. Amino acid residues 133 (TM2), 713 (TM7), and 952 (TM11), which correspond to the three bimane-labelled Cys residues in the TMDs, are shown as green spheres in (A) front view and (B) top view.
Figure 6.2 Amino acid residues in the TMDs of *C. elegans* Pgp

The crystal structure of *C. elegans* Pgp (PDB: 4F4C) is shown in ribbon format, with the N-terminal half TMD1 in green and the C-terminal TMD2 in orange. Amino acid residues L161 (TM2), T760 (TM7), and C996 (TM11), which correspond to the three Cys residues in the TMDs of mouse Pgp, are shown as purple spheres in (A) front view and (B) top view.
6.2 Suggestions for future work

The binding affinity of labelled Pgp for various substrates could be profiled using bimane fluorescence. We expect that bimane could be a powerful extrinsic fluorescent probe because of the location of three bimanes close to the substrate-binding pocket. This would allow binding affinity determination for substrates that are not compatible with MIANS or Trp fluorescence. In addition, $K_d$ values could be determined for substrates exhibiting low levels of Trp quenching, since bimane shows higher levels of fluorescence quenching, thus giving a higher accuracy.

A study by Wen et al. proposed that the TMDs of Pgp are more flexible than those of other ABC exporters (105). In the simulation, they discovered that the NBDs separated wider than in the crystal structure, and a lipid molecule partially entered the lumen, indicating a pathway for drug to directly partition into the pocket from the membrane. Labelling Pgp with a thiol-reactive agent that has a different size to bimane would provide interesting information on how the size of drug occupying the substrate-binding pocket affects the ATPase activity, drug binding, and drug transport function. The interference from a larger molecule linked to the pocket with H- or R-site substrate binding would give us a clue to the flexibility of the substrate-binding pocket. Several large thiol-reactive fluorescent dyes, including BODIPY TMR cadaverine IA, BODIPY Fl C1-IA (106), and a novel dye (known as probe 3 (107)), have been characterized. All of them can label Cys residues in proteins with high specificity and show little or no non-specific labelling at very high concentration. It is possible that the binding pocket
might be able to accommodate a molecule larger than bimane and still have very little effect on binding affinity and transport function. This could be good evidence to show how flexible the binding pocket is.

In the presence of ADP, only 5 Cys residues are labelled by PDT-bimane, three residues in the TMDs and two residues in the NBDs that are distant from the catalytic site. Two of the Cys residues in the TMDs of hamster Pgp are located in the upper part of the substrate-binding pocket (C714 and C953), which could be involved in binding of the cyclic peptides QZ59-RRR and QZ59-SSS. It is possible that when QZ59-RRR and QZ59-SSS occupy the binding pocket, they can prevent those Cys residues from PDT-bimane labelling, thus reducing the stoichiometry of labelling. Additionally, other Pgp substrates could be used to see whether any of them can block bimane labelling. This would be useful information to define the overlap between the sub-sites for substrates and bimane.
References


