P-Glycoprotein Does Not Reduce Substrate Concentration from the Extracellular Leaflet of the Plasma Membrane in Living Cells

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ABSTRACT

P-glycoprotein (Pgp), a member of the ATP-binding cassette family of transporters, is an important mediator of multidrug resistance in cancer. Pgp exhibits a very broad specificity for substrates. These substrates share a common feature of being amphipathic and can orient into either leaflet of the membrane bilayer. Current evidence suggests that Pgp recognizes and extracts substrates from the membrane bilayer, but from which leaflet is unresolved. To directly test whether Pgp can decrease substrate concentration in the extracellular leaflet of the plasma membrane in living cells, we used the fluorescent lipid analogue 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH). TMA-DPH in the extracellular solution rapidly partitions into the extracellular leaflet of the plasma membrane and exhibits slow transbilayer flipping into the cytoplasmic leaflet. Because TMA-DPH fluorescence is confined to the extracellular leaflet in early time points after addition but labels intracellular membranes after longer incubation, we can assess the effect of Pgp on TMA-DPH concentration from both extracellular leaflet and intracellular membranes. Transient transfection with a Pgp and the green fluorescence protein (GFP) fusion protein generated cells with heterogeneous expression levels of Pgp-GFP. Compared with nonexpressing cells, cells expressing Pgp-GFP showed decreased accumulation of TMA-DPH in intracellular membranes but similar levels of accumulation in the extracellular leaflet of the plasma membrane. Additionally, in drug-selected MCF7/ADR cells, which constitutively express high levels of Pgp, inhibition of Pgp by cyclosporin A resulted in significantly increased accumulation of TMA-DPH in intracellular membranes but no difference in its accumulation in the extracellular leaflet of the plasma membrane. These data indicate that whereas Pgp can extract TMA-DPH from the cytoplasmic leaflet of the membrane, any activity Pgp may possess in the extracellular leaflet is insufficient to decrease TMA-DPH concentration there and, therefore, does not contribute to lowering the cellular levels. Pgp is the prototype of an increasing number of clinically important ATP-binding cassette transporters of amphipathic drugs and lipids. These results may help decipher a common mechanism of these transporters.

INTRODUCTION

The development of MDR is the largest impediment in the chemotherapeutic treatment of cancers (1–3). Pgp, a plasma membrane ABC protein, is the prototype of many ABC proteins involved in MDR (4–8). Expression of Pgp alone causes resistance to a variety of structurally and mechanistically diverse chemotherapeutic agents (9, 10). Current evidence indicates that Pgp mediates resistance by the active, ATP-dependent, efflux of drugs from the plasma membrane of cells. However, how Pgp can exhibit such broad specificity for diverse compounds remains poorly elucidated (11, 12).

Pgp has been proposed to recognize its substrates within the lipid bilayer (4, 11, 12). This is based on the observation that most substrates, which include short chain lipids (13), are amphipathic and have a high propensity to partition into the bilayer. The concentration of these substrates is generally several orders of magnitude higher in the bilayer than in solution (14–17). Substrate accumulation in the membrane may allow Pgp to recognize a wide spectrum of substrates with low affinity using a nonspecific hydrophobic-binding pocket (18). Many observations support this hypothesis. Photo-cross-linking and mutational analysis indicate that the transmembrane domains of Pgp form the interaction sites for substrates (19–22), and Pgp activity can decrease the concentration of substrates in the membrane (23).

It remains to be resolved whether Pgp extracts substrates from the cytoplasmic, extracellular, or both leaflets of the plasma membrane. One model posits that the primary function of Pgp is to extract substrates from the membrane to the extracellular aqueous phase in what has been named the “vacuum-cleaner” hypothesis (11, 24). In this model Pgp could be recognizing substrates in either leaflet. An alternative model posits that the primary function of Pgp is to transport substrates from the cytoplasmic leaflet to the extracellular aqueous phase or extracellular leaflet (25, 26). In this model Pgp would engage substrates in the cytoplasmic leaflet.

To determine where Pgp can recognize substrates, we used TMA-DPH, a fluorescent compound of which the concentration can be directly measured in the extracellular leaflet of the plasma membrane of living cells. Using drug naïve cells transfected with a Pgp-GFP fusion protein and MDR cells constitutively expressing wild-type Pgp, we determined the effect of Pgp activity on both intracellular and extracellular leaflet concentration of TMA-DPH.

MATERIALS AND METHODS

Tissue Culture and Transfection. MCF-7 and MCF-7/Adr cells were maintained in MEM (Cellogel, Herndon, VA) with 10 μg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO) and 10% fetal bovine serum (Hyclone, Logan, UT; Refs. 27, 28). MCF-7/Adr cells were selected continuously in 0.8 μM of doxorubicin (Calbiochem, La Jolla, CA). HeLa cells were maintained in DMEM (Cellogel) with 10% bovine calf serum (Hyclone). Transient transfection of HeLa cells was performed as described previously using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN; Ref. 10).

Fluorescent Microscopy. Fluorescence microscopy was done on an inverted IX-70 microscope using either a 60 × 1.2 numerical aperture water objective or a 100 × 1.35 numerical aperture oil objective (Olympus America, Melville, NY). Images were collected using a cooled charged coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan), a National Instruments MAQ-1424 digital image acquisition card, and in-house software written in LabVIEW (National Instruments, Austin, TX). Excitation was provided using a 150 W Xenon arc lamp (OptiQuip, Beaver Falls, PA). Excitation and emission wavelengths were selected using filter wheels (Ludl Electronic Products, Hawthorne, NY) and all of the filters were from Chroma Technology (Brattleboro, VT). Rhodamine 123, Alexa 594 transferrin (Alexa-Tfn), Texas Red 10 kDa-dextran, and TMA-DPH were from Molecular Probes (Eugene, Oregon). The following filters sets were used: GFP, rhodamine 123, λem = 480–490 nm and λex = 500–550 nm; TMA-DPH, λem = 340–380 nm and λex = 430–470 nm; and Alexa-Tfn and Texas Red dextran, λem = 540–580 nm and λex = 600 nm Longpass. Multicolor images were acquired as...
multiple monochrome images using appropriate filters and merged into a pseudocolor image with TMA-DPH as blue, GFP or rhodamine 123 as green, and Alexa-Tfn as red.

At least 12 h before microscopy, cells were plated on Labtek coverglass chambers (Naperville, IL) precoated with 10 μg/ml fibronectin (Life Technologies, Inc., Rockville, MD). For analysis of plasma membrane staining of TMA-DPH, cells were suspended in 1 μM of TMA-DPH in OptiMEM I reduced serum medium without phenol red (Life Technologies, Inc.) prewarmed to 37°C and immediately observed by microscopy without change of medium. For intracellular staining of TMA-DPH, cells were incubated for 10 min with 1 μM of TMA-DPH, washed 3 × with prewarmed 37°C PBS, and incubated for an additional period of time in dye-free OptiMEM before microscopy. For double labeling of intracellular TMA-DPH and rhodamine 123, cells were incubated with TMA-DPH, washed, and incubated in dye-free OptiMEM as above; additionally, 100 nM of rhodamine 123 was included in the dye-free washout OptiMEM. For triple labeling of intracellular TMA-DPH, rhodamine 123, and Alexa-Tfn cells were done similar to double labeling above, except cells were incubated with 100 μg/ml Alexa-Tfn and 1 μM of TMA-DPH for 10 min initially and washed for 3 × with ice-cold 140 mM NaCl, 10 mM Na-2-(N-morpholino)ethanesulfonic acid (pH 5.0) to remove plasma membrane Alexa-Tfn (29) before the PBS wash. For some experiments, 10 μM of cyclosporin A (Calbiochem) was added during the labeling and/or washout.

**FACS Analysis.** FACS data were acquired on a FACS Vantage using CellQuest software (Becton Dickinson, San Jose, CA), which has 352 nm and 488 nm Argon laser lines for excitation of TMA-DPH and GFP, respectively. Compensation for fluorescence overlap was adjusted using cells labeled with a single fluorophore. Pgp-GFP-transfected HeLa cells were nonenzymatically dissociated using Cell Stripper (Cellgro) and resuspended in OptiMEM before TMA-DPH labeling and FACS analysis. For quantification of plasma membrane TMA-DPH accumulation, cells were placed in prewarmed OptiMEM with 1 μM of TMA-DPH and immediately run in FACS. For quantification of intracellular TMA-DPH, cells were incubated with 1 μM of TMA-DPH for 10 min, washed, and incubated in dye-free medium for 30 min before FACS acquisition. Immediately before image acquisition, cells were harvested and resuspended in ice-cold PBS.

**RESULTS**

**Experimental Design.** To test the ability of Pgp to extract substrates directly from the extracellular leaflet of the plasma membrane in living cells, we used the fluorescent lipid analogue TMA-DPH (Fig. 1A). TMA-DPH has several properties that make it particularly useful (30, 31):

1. TMA-DPH can embed into either leaflet of the membrane bilayer. It is fluorescent when embedded in the membrane bilayer and nonfluorescent in the aqueous medium. Therefore, the entire fluorescence of a sample with TMA-DPH in the buffer is from the membranes (Fig. 1B).

2. Because of its short lipid tail, equilibration of TMA-DPH between the extracellular leaflet of the plasma membrane and the external buffer is completed within 1 s. Washing the cells with dye-free buffer for 1 min removes >95% of the dye on the external leaflet of the plasma membrane (32).

3. Once incorporated into the external leaflet of the plasma membrane, TMA-DPH can be internalized by endocytosis and serves as a useful marker for endocytic compartments. Because of the constitutive positive charge, the dye does not quickly flip-flop between leaflets. Previous data indicate that, in L929 mouse fibroblasts, TMA-DPH does not flip into the cytoplasmic leaflet from either the external leaflet of the plasma membrane or the internal leaflet of the endocytic compartments. However, other cell types can internalize the dye into the cytoplasm either through cationic transporters or biophysical membrane differences (30).

4. TMA-DPH is a substrate for the LmrA *Lactobacillus* multidrug transporter (33).

Because TMA-DPH is a substrate of the functionally homologous LmrA, we suspected that it might be a Pgp substrate. To assess this possibility, the dye must enter the cytoplasmic compartment and label intracellular membranes. This would permit us to assay the effect of Pgp on cytoplasmic accumulation. Because of the first three properties outlined above, all of the TMA-DPH fluorescence after initial addition of the dye to cells originates from the extracellular leaflet of the plasma membrane (Fig. 1B). The intensity of this fluorescence correlates with the concentration of TMA-DPH in the extracellular leaflet. Thus, we can ask if Pgp can decrease the concentration of TMA-DPH on the extracellular leaflet of the plasma membrane with the drug present in the medium.

**TMA-DPH Enters the Cytoplasmic Compartment of HeLa Cells.** Previous data suggested that TMA-DPH would flip into the cytoplasm in some cells and stay in the endocytic compartment in others. When HeLa cells were incubated for 10 s with 1 μM of TMA-DPH at 37°C and examined with the dye still in buffer, fluorescence was confined to the plasma membrane (Fig. 1B). Because TMA-DPH rapidly partitions between lipid and solution, the confinement of fluorescence to the plasma membrane with no redistribution to the more abundant intracellular membranes implies that the dye was localized to the extracellular leaflet. Immediate washout of TMA-DPH after a 10-s incubation removes almost all of the TMA-DPH fluorescence, additionally implicating the extracellular leaflet for its localization (data not shown).

Next, we asked whether TMA-DPH could enter the cytoplasm of HeLa cells after a longer incubation. Cells were incubated in 1 μM of TMA-DPH for 10 min, washed, and resuspended in dye-free buffer for 10 min to eliminate the plasma membrane fluorescence. There was some remaining fluorescence, which was from intracellular membranes (Fig. 2A). Many intracellular structures are labeled with the strongest labeling in the perinuclear area. Notably, many long tubular...
structures reminiscent of mitochondria as well as some punctate vesicular structures were labeled. To identify these structures, cells were coincubated with the mitochondria dye rhodamine 123 and the endosomal dye Alexa-Tfn (29, 34). Rhodamine 123 labeled the tubular mitochondria with the highest density in the perinuclear region and extending to the cell periphery (Fig. 2B). All of the structures labeled by rhodamine 123 were also labeled with TMA-DPH (Fig. 2D), indicating that TMA-DPH was present in the mitochondrial membranes. Alexa-Tfn is a marker for receptor-mediated endocytosis and labeled mostly vesicular structures in the perinuclear region (Fig. 2C). Some but not all of the Alexa-Tfn labeled vesicles were also labeled with TMA-DPH (Fig. 2D). This likely represents endocytosed TMA-DPH that did not escape into the cytoplasmic compartment. When cells were incubated in dye-free medium for 120 min after the 10-min loading, there was no substantial change in either intensity or localization of intracellular TMA-DPH staining (Fig. 2E–H). This indicates that after TMA-DPH has entered the cytoplasmic compartment, the dye does not quickly diffuse from cells.

The fact that TMA-DPH labeled mitochondria of HeLa cells indicates that the dye has entered the cytoplasm, either by flipping in the plasma membrane or by escaping the endocytic compartments. The predominant labeling of the mitochondria compared with other abundant membranes such as the endoplasmic reticulum probably results from the membrane potential driven flipping of the cationic dye into the mitochondrial lumen.

**Effect of Pgp-GFP Fusion Protein Expression on TMA-DPH Accumulation.** To study the effect of Pgp expression on the localization and accumulation of TMA-DPH, we used transient transfection of the Pgp-GFP fusion protein, which generated cells that differ in expression of the fusion protein. Thus, we can specifically assay the effects of Pgp expression without the confounding factors of drug selection and clonal expansion (10).

To ask whether Pgp-GFP expression can decrease the concentration of TMA-DPH on the extracellular leaflet of the plasma membrane, HeLa cells transfected with Pgp-GFP were resuspended in 1 μM of TMA-DPH at 37°C and immediately examined with fluorescence microscopy for GFP and TMA-DPH fluorescence. TMA-DPH fluorescence was localized on the plasma membrane (Fig. 3B). In this microscope field, there were two Pgp-GFP-expressing cells, surrounded by other nonexpressing cells (Fig. 3A). The intensity of TMA-DPH fluorescence of Pgp-GFP expressing cells is not significantly different from that of nonexpressing cells (Fig. 3, B and C). There exists considerable heterogeneity in TMA-DPH staining intensity on the plasma membrane, likely attributable to cell height, which complicates definitive analysis (Figs. 1B, 3B, and 5, B and D). Nevertheless, when examining fields of cells for TMA-DPH fluorescence, it was not possible to pick out Pgp-GFP-expressing cells. This suggests that expression of Pgp does not decrease the concentration of TMA-DPH on the external leaflet of the plasma membrane.

Next, to assay whether TMA-DPH is a Pgp substrate, Pgp-GFP transfected HeLa cells were incubated with 1 μM of TMA-DPH for 10 min to allow the dye to enter the cells, then the cells were washed, and incubated in dye-free buffer for 30 min to remove all of the dye from the external leaflet of the plasma membrane. Non-Pgp-GFP-expressing cells exhibited strong cellular TMA-DPH fluorescence, whereas the Pgp-GFP-expressing cells showed significantly decreased TMA-DPH fluorescence (Fig. 3, D–F). The remaining dim TMA-DPH fluorescence of Pgp-GFP-expressing cells was localized to the perinuclear region. This staining is likely attributable to dye that has not escaped the endocytic compartments. To ensure that the decrease in intracellular TMA-DPH was not attributable to decreased endocytosis, we examined the effect of Pgp-GFP expression on Texas Red dextran uptake and found no difference (data not shown). To ensure that it was the Pgp activity of Pgp-GFP-expressing cells that caused the decrease in TMA-DPH labeling, the Pgp inhibitor cyclosporin A (10 μM) was included in the incubation and wash buffer to inhibit Pgp activity. This abolished the effect of Pgp-GFP expression on TMA-DPH accumulation (Fig. 3, G–I). To show that the Pgp-mediated decrease of TMA-DPH in the cytoplasmic compartment was attributable to efflux and not by inhibition of the pathway that allows TMA-DPH to enter the cytoplasm, both Pgp-GFP-expressing and nonexpressing cells were loaded with TMA-DPH into the cytoplasm.
Fig. 3. Effect of Pgp-GFP expression on TMA-DPH accumulation. A–G, Pgp-GFP transfected HeLa cells were resuspended in prewarmed 1 μM of TMA-DPH and immediately observed for GFP (A) and TMA-DPH fluorescence (B). There was no significant difference in plasma membrane TMA-DPH staining between the Pgp-GFP-expressing and -nonexpressing cells (C). D–F, cells were incubated for 10 min with 1 μM of TMA-DPH, washed, and then incubated for 30 min in dye-free medium before microscopy. There was significantly decreased cytoplasmic TMA-DPH labeling in the Pgp-GFP-expressing cell. This indicates that expression of Pgp-GFP decreases the accumulation of TMA-DPH in the cytoplasmic compartment. G–I, cells were incubated for 10 min with 1 μM of TMA-DPH and 10 μM of cyclosporin A, washed, and then incubated for 30 min with 10 μM of cyclosporin A in washout medium. The Pgp-GFP expressing cells showed much TMA-DPH cytoplasmic labeling as nonexpressing cells. Thus, inhibition of Pgp activity restores accumulation of TMA-DPH in Pgp-GFP-expressing cells. J–L, cells were incubated for 10 min with 1 μM of TMA-DPH and 10 μM of cyclosporin A, washed, and then incubated for 30 min with 10 μM of cyclosporin A, as above. Next, cells were additionally incubated in cyclosporin A-free medium for 30 min. The cells that express Pgp-GFP showed decreased TMA-DPH accumulation. This implies that Pgp-GFP mediates efflux of TMA-DPH. Scale bar is 20 μm.

Fig. 4. FACS analysis of Pgp-GFP and TMA-DPH. A, Pgp-GFP transfected HeLa cells were resuspended in prewarmed 1 μM of TMA-DPH and immediately processed by FACS. The TMA-DPH fluorescence of cells at this time is predominantly from the extracellular leaflet of the plasma membrane. This panel shows a scatter plot of cells with total cellular GFP fluorescence on the X axis and TMA-DPH fluorescence on the Y axis. Cells with <10^1 GFP fluorescence units are nonexpressing (from analysis of nontransfected cells), and cells with >10^4 units show different levels of expression. The TMA-DPH fluorescence of cells is not significantly different in cells exhibiting different levels of GFP fluorescence, implying that expression of Pgp-GFP does not affect the TMA-DPH concentration on the extracellular leaflet of the plasma membrane. B, cells were incubated for 10 min with 1 μM of TMA-DPH, washed, and incubated in dye-free medium for 30 min. The TMA-DPH fluorescence of cells at this time is cytoplasmic. Nonexpressing cells exhibited an average TMA-DPH fluorescence of ~2 × 10^4 units. Here, cells with increasing GFP fluorescence showed decreasing TMA-DPH fluorescence, where cells with the highest GFP fluorescence exhibited an average TMA-DPH fluorescence of ~4 × 10^4 units. This indicates that increasing Pgp-GFP expression results in decreasing cytoplasmic TMA-DPH accumulation.
These data indicate that a significant percentage of the TMA-DPH had flipped into the cytoplasm and was extruded by Pgp.

Effect of Pgp Inhibition on TMA-DPH Accumulation in Drug-selected MDR Cells. To ensure that the introduction of GFP to the COOH terminus of Pgp did not alter the activity profile of the wild-type enzyme such as inhibit activity on substrates from the extracellular leaflet while preserving activity on substrates from the cytoplasmic leaflet, we analyzed wild-type Pgp using the MDR MCF-7/Adr breast cancer cells, which overexpress wild-type Pgp (35, 36). We examined the effect of Pgp inhibition with cyclosporin A on TMA-DPH accumulation. Cells were incubated with 1 μM of TMA-DPH with or without 10 μM of cyclosporin A and immediately examined by microscopy to quantify labeling of the extracellular leaflet of the plasma membrane. There was no significant difference in this initial labeling intensity of TMA-DPH in the presence or absence of cyclosporin A (Fig. 5). Next, MCF7/Adr cells were incubated for 10 min with 1 μM of TMA-DPH and washed for 30 min in TMA-DPH-free buffer containing 100 nM of rhodamine 123, all in the presence or absence of cyclosporin A. There was a dramatic difference in both the intensity and localization of TMA-DPH fluorescence (Fig. 6). In the absence of cyclosporin A, there was very dim perinuclear staining and no detectable rhodamine 123 staining, confirming the high activity of Pgp in these cells (Fig. 6, A–C). When cells were incubated with cyclosporin A, TMA-DPH labeled the perinuclear area as well as spherical organelles of ~1 μm in diameter. These same structures were also labeled by rhodamine 123, confirming that they are mitochondria, which are spherical and not tubular in these cells (Fig. 6, D–F). This data suggest that, like Pgp-GFP, wild-type Pgp also cannot decrease the concentration of TMA-DPH on the external leaflet of the plasma membrane but does extrude TMA-DPH from the cytoplasmic compartment.

DISCUSSION

To enter a cell, amphipathic drugs first rapidly partition into the extracellular leaflet of the membrane then flip into the cytoplasmic leaflet and finally partition into the cytoplasm (16, 26). The slow rate at which these drugs flip between the membrane leaflets results in a pseudoequilibrium between the extracellular solution and extracellular leaflet, and between the cytoplasmic compartment and cytoplasmic leaflet. A Pgp-mediated decrease in drug concentration from the extracellular leaflet would decrease the drug available for “flipping” from the extracellular leaflet to the cytoplasmic leaflet. Kinetically, the rate of accumulation would mimic that of decreasing the extracellular drug concentration. In contrast, a Pgp-mediated decrease in the cytoplasmic leaflet would increase the net efflux of drug.

A number of experimental observations are consistent with the vacuum-cleaner hypothesis in which Pgp engages substrates from either leaflet. Firstly, kinetic studies have consistently shown that MDR cells have a decreased rate of initial drug accumulation compared with drug-sensitive cells (37, 38). Because the initial accumulation rate should reflect only the influx rate, these kinetic data imply that Pgp does not only cause increased efflux but also decreased influx. Decreased concentration of substrate in the extracellular leaflet would result in decreased influx. A simple model of drug accumulation assuming that Pgp can decrease the substrate concentration from both leaflets shows a good quantitative fit to experimental data (11). Secondly, MDR cells show decreased accumulation of cleaved AM esters. On entering the cell, AM esters are cleaved rapidly by cellular esterases into nonpermeant products. This suggests that these esters never gain access to the cytoplasmic compartment (39). However, an
important caveat is that esterase activity and not AM ester influx is the limiting factor in the rate of AM ester cleavage (40). Thus, AM esters may survive in the cell for a sufficient time before hydrolysis to allow Pgp to export them. Finally, the lipophilic membrane probe $5\cdot125^\text{I}$iodonaphthalene-1-azide can accept energy from the fluorescent substrate doxorubicin and then label proteins in the immediate vicinity. When drug-sensitive cells were exposed to $5\cdot125^\text{I}$iodonaphthalene-1-azide and doxorubicin, and doxorubicin was excited, many membrane proteins were labeled. However, in a MDR cell line, Pgp alone accounted for $\sim$60% of all of the labeled proteins, and labeling of other proteins was concomitantly decreased. This result suggested that Pgp activity removes substrates from the proximity of other membrane proteins, i.e., both leaflets of the membrane (24). However, since doxorubicin labels many intracellular membranes, which is more abundant than the plasma membrane, decreased intracellular concentration without decreased extracellular leaflet concentration may be enough to produce drastically diminished labeling.

Other experimental observations are consistent with the hypothesis in which Pgp extracts substrates only from the cytoplasmic leaflet of the plasma membrane. Hoechst 33342 is a Pgp substrate, which is fluorescent only when embedded in the vesicle membrane and not in solution. In inside-out plasma membrane vesicles, Pgp activity decreased Hoechst 33342 fluorescence indicating that it was extracted directly from membranes (23). However, the longer the time lag between addition of Hoechst 33342 and addition of ATP to activate Pgp, the less the decrease of Hoechst 33342 fluorescence. Because the lag time allowed externally added dye to flip to the luminal (extracellular) leaflet, this result suggested that Pgp extracts substrates from the outer (cytoplasmic) leaflet but not from the luminal leaflet. Additionally, when a fluorescent lipid that can accept FRET from Hoechst 33342 was specifically embedded in the cytoplasmic leaflet, Pgp activity caused the FRET to decrease faster than the Hoechst 33342 fluorescence (26). Whereas this experiment was taken to additionally suggest that Pgp extracts substrates only from the cytoplasmic leaflet, it is complicated by the important caveat that FRET is highly distance-dependent (sixth power), and any decrease in concentration in a greater decrease in FRET.

Our data indicate that Pgp does not decrease the concentration of one substrate, TMA-DPH, in the extracellular leaflet of the plasma membrane. While this does not necessarily indicate that Pgp cannot extract the dye from the extracellular leaflet, it does imply that any extraction must be significantly slower than the rapid equilibrium between the solution and the extracellular leaflet. More importantly, because the concentration in the extracellular leaflet determines the amount of drug that can enter the cell, Pgp must be able to decrease drug concentration from the extracellular leaflet to affect influx rates (11).

The inability of Pgp to affect TMA-DPH concentration on the extracellular leaflet is consistent with known biophysical properties of TMA-DPH. This dye partitions into lipids extremely rapidly, faster than can be measured. Using a rate of approximately several seconds for it to partition from membranes (31) and a partition coefficient of $10^3$ (33), one can estimate that TMA-DPH partitions into membranes on the order of microseconds. This is orders of magnitude higher than any estimated rates of Pgp turnover (over $10^4$). The rapid partitioning into lipids is not unique to TMA-DPH but is shared by most Pgp substrates. Using measured rates of partitioning and flipping of doxorubicin, a mathematical model showed that extraction from the extracellular leaflet of the plasma membrane at known rates of Pgp turnover has no effect on accumulation of the drug (12). Thus, extraction from the extracellular leaflet may not be an evolutionarily rational mechanism for amphiphilic substrates.

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