Published in final edited form as:

Methods Mol Biol. 2010 ; 596: 123–139. doi:10.1007/978-1-60761-416-6_7.

Flow Cytometric Evaluation of Multidrug Resistance Proteins

Adorjan Aszalos¹, Barbara J. Taylor²

¹Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, USA

²Laboratory of Cancer Biology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, USA

Abstract

There are several ways to detect proteins on cells. One quite frequently used method is flow cytometry. This method needs fluorescently labeled antibodies that can attach selectively to the protein to be investigated for flow cytometric detection. Flow cytometry scans individual cells, virtually without their surrounding liquid, and can scan many cells in a very short time. Because of this advantage of flow cytometry, it was adapted to investigate transport proteins on normal and cancerous human cells and cell lines. These transport proteins play important roles in human metabolism. Absorption in the intestine, excretion at the kidney, protection of the CNS compartment and the fetus from xenobiotics, and other vital functions depend on these transporters. However, several transporters are overexpressed in cancer cells. These overexpressed transporters pump out anticancer drugs from the cells and prevent their curative effects. The detection and quantitation of these types of transporters in cancer cells is important for this reason. Here, we review literature on flow cytometric detection of the three most studied transporters: P-glycoprotein, multidrug resistance-associated proteins, and breast cancer resistance protein.

Keywords

Transport proteins; P-glycoprotein; Multidrug resistance protein; Breast cancer resistance protein; Flow cytometer

1. Introduction

Flow cytometric detection and evaluation of three types of transport proteins will be discussed in this chapter: P-glycoprotein (Pgp, ABCB1), multidrug resistance-associated proteins (MRPs, ABCCs), and breast cancer resistance protein (BCRP, ABCG2). These transport proteins play normal physiological roles in the human body but also cause resistance to cancer chemotherapy. The physiological roles of these transporters are many: they include absorption of molecules in the intestine, regulating passage to the CNS compartment at the blood–brain barrier, passage of metabolites to the urine through the kidney, protecting the placenta from passing toxic agents to the fetus, and other functions at the liver, testis, and the lung. For further details on the expression of the transporters in humans, *see refs.* (1, 2). Any abnormality in the level of expression of these transporters can result in illness. One reason for resistance to cancer chemotherapy is that cancer cells overexpress some of these transporters and prevent the entry of the chemotherapeutic agents

into the cancer cells to exert their curing effects. For these reasons, the analysis of these transporters has become very important in the last two decades.

While there are other methods to analyze the presence and amount of these transporters in cells, one of the best methods to analyze, and especially quantitate transporters on the plasma membrane is flow cytometry (3). To distinguish among these transporters by flow cytometric methods, specific antibodies have been developed and specific inhibitors, or modifiers of their function, have been evaluated. Figure 7.1 shows the primary structure of Pgp and the known attachments for three surface antibodies to their epitopes. Antibodies, substrates, and modifiers are listed with the cited methodologies in the text. To distinguish among these three types of transporters, in general, the following molecules could be mentioned. Calcein AM, a fluorescent substrate, is a characteristic substrate for MRP1. Fumitremorgin C (FTC) is a specific inhibitor of BCRP. Rhodamine 123 is not specific but in combination with verapamil, a Pgp substrate, can detect Pgp. Expression of the different transporters on cells can be distinguished with the combination of these agents, as detailed in some of the methodologies described later.

2. Evaluation of Pgp by Flow Cytometry

Several research groups have established protocols for detecting Pgp by flow cytometry for reasons of interest in their laboratories. We will now detail general guidelines provided by each of the main laboratories with established protocols. Next, we describe flow cytometric investigations as applied to some particular purposes.

Broxterman et al. analyzed the conditions for determining the amount of Pgp and its functionality on acute myeloid leukemia (AML) cells by flow cytometry (4). Their general recommendation for this evaluation can be summarized as follows. One can estimate the number of Pgp molecules on AML cells by using the antibody (Ab) MRK16, which binds to a surface epitope on the Pgp molecule. Other Abs binding to surface epitopes, such as UIC-2 can also be used. Phycoerythrin (PE)-labeled Abs yield higher sensitivity than fluorescein (FITC)-labeled Abs. The amount of Pgp expressed on AML cells can be determined using cell lines with a known amount of Pgp expressed under the same experimental conditions used for the AML cells. These authors used KB cell lines (KB-3-1, KB8, and KB-8-5), which each express different amounts of Pgp. These reference cell lines should be used fresh from frozen stocks after each 3 or 4 months of culturing. These cells tend to express higher levels of Pgp after longer culturing in the presence of the selecting agents. *See Table 7.1 for flow cytometers used.*

Broxterman et al. provide some general considerations concerning the functional analysis of Pgp expressed on cells (5). Pgp functionality is measured in order to find the transport capacity of the transporters. It also helps to determine the proper modulators for a particular patient. Fluorescent probes for accumulation and efflux studies include rhodamine 123, DiOC₂(3), calcein-AM, Hoechst 33342, BCECF-AM, Furo-2-AM, Fluo-3-AM, daunorubicin, and doxorubicin. The first three are particularly suitable to study Pgp modulator molecules. All these fluorescent probes have high ratios of active to passive transport and they equilibrate between cells and the medium relatively quickly. Equilibration

of some of these probes, however, depends on certain characteristics of the cells studied, such as their membrane potential, intracellular pH, intracellular Ca^{2+} , and DNA content. It is important that the Pgp modulator does not interfere in any way, such as through fluorescence, with the fluorescence and transport of the fluorescent probe. In this regard, valspodar was found superior to cyclosporin A and verapamil. Because transport is ATP dependent, sufficient glucose should be present in the medium and the cells should be viable during the study.

A group in England led by M. Pallis adapted the Dutch protocol with a slight modification that is based on the work of the group of Broxterman et al. (5, 6). The adapted protocol was tested for expression and functionality of Pgp in AML and myelodysplastic syndrome cells in a multicenter trial in England.

Leith et al. described a method for functional assay of Pgp in AML and cells of bone marrow origin (7). Differences in the efflux of the fluorescent substrate $\text{DiOC}_2(3)$ in the presence and absence of the modulator molecule can be measured over a time course according to Krishan (8, 9). In these experiments besides the forward vs. side scatter and forward scatter vs. fluorescence, time vs. fluorescence histograms also could be generated. A multicolor analysis for peripheral blood mononuclear cells (PBMCs) was established by Ford et al. (10). The assay was established for detection of Pgp expression in subsets of blood cells of healthy people.

Feller et al. described a method to analyze Pgp in solid tumor cells (11). Depending on the origin of the cells isolated from solid tumors, the expression of Pgp on the cells as measured by flow cytometry may or may not correspond to the measured Pgp RNA. One reason for this possible discrepancy could be the heterogeneous expression of Pgp in the isolated cells.

Aszalos and Weaver described a flow cytometric test for expression of Pgp on cell lines (12). Pgp on cells can be detected with the MRK16 mAb either labeled directly with FITC according to the instructions provided with the labeling kit or by using a secondary FITC Ab. Histograms showing fluorescence intensities when MRK16-FITC, MRK16 plus a secondary FITC Ab and isotype-matched Ab plus anti-isotype FITC Ab were used are shown in Fig. 7.2. Differences in fluorescence shifts between two histograms can be evaluated by the Kolmogorov–Smirnov statistics included with the flow cytometer software. See Table 7.1 for flow cytometers used.

Numbers of Pgp molecules on clinical and in vitro drug-selected cells can be determined according to Aleman et al. (3). To determine number of Pgp molecules on cells, a series of beads with increasing numbers of fluorescein molecules is used. Standard fluorescent beads with defined numbers of fluorescein molecules were used as follows: 6,318, 15,877, 53,989, 82,914, 123,338, 170,473, 353,992, and 437,815 fluorochromes. Fluorescence intensities of the beads are obtained with a flow cytometer equipped with a 488-nm laser and a 530 emission filter. Beads are shaken well and mixed two intensities per tube for a total of four tubes. A graph is plotted from the means of individual histograms of the beads. Means of histograms obtained from 10^4 cells labeled with MRK16 plus antimouse IgG-FITC or another FITC Ab against a surface epitope of Pgp are matched against the graph. Both the

beads and the cells are suspended in the same PBS buffer. The graph for the means of the histograms of the beads is plotted on semilogarithmic paper. Alternatively, QuickCal data analysis software (Bangs Laboratories) can be used. Means of histograms of fluorescence intensities obtained from the tested cells are matched against the plot obtained with the beads. *See* Table 7.1 for flow cytometers used.

Beck et al. described a consensus recommendation for detection of Pgp in patient's tumors (13). A multinational workshop was organized for the detection of Pgp in clinical samples. The aim of this workshop was to standardize factors such as reagents, preparation of samples, detection of end-points, and methodology of analysis, in the determination of the role of Pgp in drug resistance in clinical evaluation of a patient's treatment. The following recommendations were made: Hematological samples or disaggregated solid tumor cells are best analyzed by flow cytometry when the preparation is fresh. Samples can be cryopreserved at -135°C in 20–90% fetal bovine serum with 10% DMSO. Otherwise samples can be kept on ice for 24 h before analysis. Recommended Abs, such as MRK16, UIC-2, and 4E3 are best to use because they recognize external epitopes on the Pgp molecule. The advantage of using Abs that recognize an external epitope is that in flow cytometric analysis, correlation can be made by multicolor analysis with other surface antigens and with functional measurement of Pgp using dye accumulation/efflux measurements.

For flow cytometric detection of fluorochromes attached to primary or secondary Abs, PE is preferred over FITC. The reason for this recommendation is that PE has a higher quantum efficiency and therefore detection of low levels of Pgp expression is more accurate. Also, autofluorescence is less in the PE detector (585 nm) than the FITC (530 nm), so the signal-to-noise ratio is higher with PE. An isotypically matched Ab with the same fluorochrome should be used as a baseline control.

Well-characterized cell lines with a known amount of Pgp expressed, as determined by mRNA and flow cytometric methods, should be used to validate flow cytometric assays in a particular laboratory. A cell line developed by Beck et al. (13), CEM/VLB, or the aforementioned KB cell line by Broxterman et al. could be used. For clinically relevant low-level expression of Pgp, the 8226/Dox6 cell line could be used.

Normal cells expressing a low level of Pgp should be electronically gated out from the malignant cells based on surface marker expression. Reporting results can be done by evaluation of the mean channel shift between control and sample by the Kolmogorov–Smirnov statistical method provided with most flow cytometers or by continuous variable data without a cut-off point for positivity. Drug efflux measurement is preferred to a drug accumulation test, using cyclosporin A, verapamil, or valspodar as modulators of Pgp function. For drug efflux measurements, the fluorescent substrates DiOC₂(3), rhodamine 123, Hoechst 33342 or the drugs daunorubicin and doxorubicin could be used. The dyes have more favorable uptake and efflux kinetics. The flow cytometric efflux studies should be correlated with Ab-mediated Pgp expression determination. The reason for this necessary correlation is that transporters other than Pgp, such as MRPs, may be responsible for the efflux of the substrate. The consensus report indicates that a tumor's resistance to

chemotherapy does not necessarily correlate with Pgp expression. After considering the aforementioned recommendations, one can use materials and techniques found in several of the earlier described detailed analytical methods.

Wang et al. described an assay for quantitative determination of modulation of the function of Pgp by compounds (14). The aim of this study is to quantitatively assess the ability of a compound to modulate the function of Pgp. For this purpose, increasing doses of the tested compound are used in the efflux assay and the inhibition of the function of Pgp is determined in relative % to that of ortho vanadate. While Wang et al. used the CR1R12 cell line, other cell lines exclusively expressing the transporter Pgp could be used for this study. Wang et al. found that 2 μ M daunorubicin was the optimal concentration of substrate for the cell line they used. Among the tested compounds, cyclosporin A and progesterone gave the most inhibition of Pgp function, 75 and 60%, respectively. Verapamil and terfenidine inhibition were 40–50%, relative to orthovanadate. Note that second- and third-generation Pgp modulators can achieve greater inhibition of Pgp function than the compounds tested by Wang et al. *See Table 7.1 for flow cytometers used.*

3. Evaluation of MRPs by Flow Cytometry

Several MRPs have been characterized and described in the literature. Later we detail the known flow cytometric evaluation of some of these transport proteins from laboratories involved in their particular research.

Janneh et al. evaluated the expression of Pgp and MRPs in peripheral blood mononuclear cells (PBMCs) for the purpose of determining the interaction of various protease inhibitors at the level of Pgp and several MRPs (15). In their study, they determined the uptake of (¹⁴C) lopinavir into PBMCs and some specific cell lines expressing various transporter proteins, in combination with specific inhibitors of Pgp, MRP1, MRP2, and organic anion transporter protein. Following this determination, they evaluated the modulating effect of several other protease inhibitors at the level of the transport proteins. Flow cytometry served to determine the presence of MRP1 in PBMCs, obtained from patient buffy coats. The specific modulators used by Janneh et al. (15) in their study are worth mentioning despite the fact that they did not use them in the flow cytometric experiment: tariquidar (Pgp specific), MK571 (MRP specific), frusemid (MRP1/2 specific), dipyridamole (MRP1/Pgp specific), and probenecid (MRP2/OATP specific). These specific blockers can be used to differentiate among the transport proteins for the efflux of drugs and compounds from cells. *See Table 7.2 for flow cytometers used.*

Feller et al. evaluated the expression of MRP in several cell lines (11). Their aim was to find the best combination of fluorescent substrate and modifier of the function of MRP. They came to the conclusion that the best probe to detect the specific function of MRP1 by flow cytometry is to use daunorubicin as substrate and genistein as modulator of the function of MRP1. They also concluded that genistein decreases the fluorescence of rhodamine 123 and calcein-AM in sensitive cells, and therefore these fluorescent substrates cannot be used together with the specific modulator of MRP1, genistein. They also found that valspodin and vincristine are not suitable substrates of MRP1.

Meaden et al. compared the expression of MRP in PBMCs between HIV-infected and noninfected patients (16). They concluded that the expression of MRP in PBMCs is the same in HIV-infected and noninfected patients. (Pgp expression is less in HIV infected than noninfected patients.) *See Table 7.2 for flow cytometers used.*

Braga et al. compared the expression of MRP1 and Pgp in cells expressing both transporters (17). For detection of MRP1 they used carboxy fluorescein diacetate (CFDA), and for Pgp, rhodamine 123. The CFDA is nonfluorescent as is, but is hydrolyzed in cells by esterases to the fluorescent derivative. The two probes can distinguish between Pgp and MRP1 (18), as rhodamine 123 is a substrate of Pgp and CFDA a substrate of MRP1. Braga et al. analyzed the effect of oleanolic acid on the transport properties of the two transporters. They found that oleanolic acid inhibits the function of MRP1 but not that of Pgp.

McAleer et al. studied the characteristics of MRP5 (MOAT-C, ABCC11) and determined the substrate specificity of this transporter by flow cytometry (19). A comparison was made between stably-transfected and nontransfected HEKc10 cells using probes, 5-chloromethyl fluorescein diacetate (CMFDA), fluorescein diacetate (FDA), 2',7'-bis-(2carboxyethyl)-5(and 6)-carboxy fluorescein acetoxymethyl ester (BCECF-AM), daunorubicin, tetra methyl rosamine (TMR), and calcein-AM. Flow cytometric studies indicated that CMFDA is a substrate of MRPS, but daunorubicin, calcein-AM, and TMR are not. They also found by a non-flow cytometric method, fluorometry, that FDA and BCECF-AM are also substrates of MRPS. *See Table 7.2 for flow cytometers used.*

Leidert et al. analyzed the influence of MRP2 (cMOAT, ABCC2) expression in melanoma cells on platinum and DNA adduct formation (20). They found that an inverse correlation exists between expression of MRP2 and adduct formation. In connection with this study, they performed cell cycle analysis on cisplatin-treated cells. This analysis indicated a cisplatin-triggered G₂ arrest in both sensitive and resistant cells. MRP expression was done by Northern blot and RT-PCR analyses and not by flow cytometry. One should mention in connection with this study that flow cytometry would have analyzed MRP1 expression on the cell membrane, while the Northern blot plus RT-PCR analyzed all MRPs in the membrane plus in the cytoplasm.

Other MRPs (MRP3, MRP4, MRP7, and MRP8) have been characterized in membrane vesicles and not by flow cytometry. Antibodies have been developed against two of them, MRP3 and MRP6. Provided these antibodies are available, references for MRP3 (21) and MRP6 (22) can be found in the reference section for potential use in flow cytometry.

4. Evaluation of BCRP by Flow Cytometry

BCRP is found in the human placenta, bile canaliculi, the colon, small bowel, and in brain microvessel endothelium. It is overexpressed in breast and leukemia cancer tissues. In normal tissues, this transporter protects the organs from potentially toxic xenobiotics. A complete treatment of this transport molecule, including genetics, chemistry, modulators of BCRP, transported molecules, antibodies to BCRP, mutation variants of BCRP, and physiological function was published by Doyle and Ross (23). BCRP effluxes the substrates

mitoxantrone, daunorubicin, bisantrene, prozasin, rhodamine123 (only in BCRP mutation variants), topotecan, and LysoTracker. The Pgp substrates verapamil (low dose), vinblastine, paclitaxel, and the MRP1 substrate calcein are not transported by BCRP. Based on these different transport properties, these transporters can be distinguished from one another by flow cytometry.

Any standard flow cytometer with a 488-nm laser can be used for detection of BCRP. For these studies, either a FACSCan or a one- or two-laser FACSCalibur was used. The antibodies available (5D3, BXP-21, BXP-34) can be used with FITC (FL1, 530 nm) or PE (FL2, 575 or 585 nm). Substrates transported by BCRP include topotecan, BODIPY-prazosin, pheophorbide a, and BBR 3390 (all detected in FL1, 530 nm); topotecan, daunorubicin, and doxorubicin (all detected in FL2, 575 or 585 nm); and mitoxantrone (detected in FL3, 650 or 670 nm). For increased sensitivity with mitoxantrone, a 2-laser cytometer with a 633–639 laser can be used for excitation with detection in a 660/20 filter. Flow cytometers equipped with a 488-nm laser and a 355-nm laser may be used to detect Hoechst 33342 with SP (side population) cells detected at two emission wavelengths; blue at 424/44 nm and red at 675 nm LP with the signal split by a 640-nm LP dichroic mirror. In the study cited here, a FACS Vantage was used. *See* Tables 7.3 and 7.4.

There is no unified flow cytometric experiment to detect BCRP. Therefore, experiments used to measure BCRP functions in cells for different purposes are detailed later in connection with the aims of the individual investigators. Detailed descriptions of the flow cytometers are given earlier.

A group led by a scientist at Roswell Park Cancer Institute did a basic study. Minderman et al. (24) studied the sensitivity of two antibodies (Abs) to BCRP in MCF-MX8, MCF-AdVp3000, and 8226MR20 mitoxantrone-resistant cells and in their parental cell lines. Wild type HL60, the Pgp-expressing cell line HL60/Adr, and the MRP1-expressing cell line A2780/Dx5b were included in the study. The BCRPT482 cell line (containing a mutated BCRP in which arginine 482 was replaced with threonine) was also studied.

Their aim was to determine the sensitivity of two Abs, BXP-21 and BXP-34, to two epitopes on BCRP. The detection sensitivity was assessed by flow cytometry and immunohistochemistry. The study included different fluorescent molecules as possible substrates and modulators of BCRP function. Among the different fluorescent molecules, only mitoxantrone was found to be a substrate of BCRP, and therefore its uptake and efflux were studied in the presence and absence of the BCRP modulator. The fluorescence histograms obtained with the fluorescently labeled goat anti-mouse secondary Ab were evaluated with the D parameter of the Kolmogorov–Smirnov statistical method that is included in flow cytometry software. Appropriate isotype control Abs (IgG1 and IgG2a) were used to obtain baseline fluorescence. Excitation was at 488-nm wavelengths with an argon laser and detection at 530 nm for the FITC-conjugated Abs. To evaluate the mitoxantrone concentration in cells, two different excitation wavelengths were used: a 635-nm red diode laser with a 661-nm emission filter and a 488-nm laser with a 670-nm emission filter.

The two Abs bind only at internal epitopes. For this reason, cells had to be fixed in formaldehyde for 10 min at RT followed by 90% methanol treatment for 10 min to permeabilize the plasma membrane. Cells were then incubated with the primary Abs or the isotype Abs at 4°C for 60 min. After washing with PBS with 0.01% Tween, cells were incubated with the secondary fluorescent Ab for 20 min at 4°C.

Results of the flow cytometry indicated that the two Abs bind only to wild type or mutated BCRP but not to Pgp or MRP1 or lung resistant protein (LRP) in cells. The presence of BCRP was verified by statistical evaluation when the *D* value was higher than 0.2. This cut off point was in agreement with similar flow cytometric analysis for the positive expression of MRP1 and LRP when cells must be permeabilized for Ab binding to internal epitopes (7). Experiments with both primary Abs resulted in qualitatively the same results, although the *D* values varied somewhat. Interestingly, the mutant BCRP in 8226/MR20 cells could be detected with flow cytometry with the two Abs but not with immunohistochemistry.

Uptake and efflux of mitoxantrone, a substrate of BCRP, was studied with or without modulator molecules. Using excitation at 488 nm and emission at >670 nm was sensitive enough to detect mitoxantrone in cells with low expression of BCRP. A mitoxantrone concentration of 0.01 μ M is required for the detection with excitation of the 488-nm laser and emission at 635 nm. Efflux studies were done with the substrates mitoxantrone, DiOC₂ (3), rhodamine 123, and doxorubicin in Pgp, MRP1, and wild-type and mutant BCRP-expressing cells. The efflux difference between measurements in the presence or absence of transporter modulators was recorded. The modulator for Pgp was valspodar 2.5 μ M, for MRP1 probenecid 1 mM, and for BCRP fumitremorgin C (FTC) 10 μ M. *D* values were calculated by the Kolmogorov–Smirnov method from histograms obtained with or without the appropriate modulator. The *D* values were indicative of the extent of modulation of efflux of substrates by the applied modulator of the efflux pump. For example, the *D* value with mitoxantrone was above 0.29 for all transporter molecules in each cell (on a scale of 0–1), indicating that the selected modulators blocked the function of all the transporters and that mitoxantrone is transported by all these transporters. Contrary to this, *D* values indicated that efflux of rhodamine 123 was substantially blocked in Pgp- and MRP1-expressing cells but only slightly inhibited in BCRP-expressing cells with the appropriate modulators.

The method developed by Minderman et al. is well suited to analyzing clinical samples for expression of BCRP because a flow cytometer equipped with the standard 488-nm argon laser could be used (24). Detection of BCRP with the two available Abs would indicate the presence of BCRP even in cells with low levels of expression. Measuring efflux with mitoxantrone can indicate the extent of expression when FTC, a selective modulator of BCRP, is used.

Another flow cytometric method was worked out by Kawabata et al. for detecting BCRP in clinical tissues with various levels of the BCRP mRNA (25). The aim of their investigation was to assess how much BCRP expression constitutes drug resistance in lung cancer. They measured BCRP mRNA levels in cell lines known to express various levels of BCRP. They then measured topotecan retention in the same cell lines by flow cytometry and correlated

the results of the two methods. The cell lines they used were PC-6/SN2-5, PC-6, NCI-H460, NCI-H441, NCI-H358, and NCI-H69. PC-6 and NCI-H69 did not efflux much topotecan, indicating low levels of BCRP expression. PL-6/SN1-5 effluxed much more topotecan, indicating high levels of expression of BCRP. The other cell lines demonstrated intermediate levels of efflux. Flow cytometry measurements by Kawabata et al. used topotecan as a fluorescent substrate of BCRP because topotecan is a good substrate of even mutant BCRPs. Kawabata et al. obtained excellent correlation between BCRP mRNA expression, as determined by real-time RT-PCR analysis, and fluorescence intensity of cells after efflux of topotecan as measured by flow cytometry. They selected NCI-H441 from among the cell lines as borderline BCRP-expressing cells, as the amount of BCRP in these cells is the amount necessary to confer drug resistance. After establishing this correlation and establishing this base line expression of BCRP, 23 nontreated non-small cell lung carcinoma tissues were examined by the two methods for BCRP expression. Their results indicated that 22% of the tumor tissues had higher efflux-related resistance than the NCI-H441 cells, conferring transport protein-based drug resistance.

Rachel Ee and colleagues used flow cytometry to assess the results of small interfering RNA (siRNA) treatment of BCRP-expressing cells to suppress the expression of this transport protein (26). They used essentially the same system as Kawabata et al., detailed earlier. They used topotecan as a fluorescent substrate, and its accumulation in cells was assessed by a FACSCalibur instrument using 488-nm excitation wavelength. BeWo choriocarcinoma cells, treated or nontreated with siRNA for 24 h, were incubated with 30 μ M/L topotecan for 15 min at 37°C and flow cytometric measurement followed.

Rabindran et al. examined the effect of fumitremorgin C (FTC) on the retention of the dye BBR 3390, 5 μ M and daunorubicin, 1 μ M, in BCRP-expressing MCF-7 cells (27).

An interesting study was done by Scharenberg et al. who analyzed the extent of expression of BCRP, Pgp, and MRP1 in lung carcinoma A549, human embryonic kidney HEK293, and several human leukemia cell lines (28). The aim of their study was to characterize the efflux protein expression in a “side population” (SP) of the cell lines as indicated by Hoechst 33342 dye retention and efflux patterns. Hoechst dye excluding cells sorted out from bone marrow and other tissues contain immature stem cells, isolated as CD34⁺/CD38⁻ or CD34⁺/KDR⁺ cells. They wanted to determine how many of these stem cells were among the studied cell lines and what type of transport proteins are expressed in them. To answer these questions, flow cytometric efflux studies were done with the fluorescent Hoechst dye and efflux protein modulators probenecid, verapamil, and FTC to determine the possible existence of the three efflux pumps. They found that FTC blocks most efflux of Hoechst dye, indicating that BCRP is the predominant transporter in A549 cells. RT-PCR experiments supported these findings. They also found that the two other transporters, Pgp and MRP1 are also expressed, but in minor quantities. A characteristic UV–red vs. UV–blue chart indicates the presence of stem cells expressing BCRP (Fig. 7.3).

BCRP has several variants. The V12M and Q141K variants were described by Zamber et al. (29). Three other variants, I206L, N590Y, and D620N, were studied by Vethanayagam et al. for their expression levels and functionalities (30). Expression levels were determined by

immunoblotting and functionality by efflux measurements with flow cytometry. All three variants transported mitoxantrone, pheophorbide a, and BODIPY-prazosin. Doyle et al. showed that the resistance of BCRP-expressing cells could be partially reversed by the antibiotic novobiocin (31). In their studies, they used the “comparative growth assay” system developed by Hausner et al. (32).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. We would like to thank Dr. Michael Gottesman for his hospitality and encouragement and George Leiman for editorial assistance.

References

- Schinkel AH, Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* 55:3–29 [PubMed: 12535572]
- Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM (2006) Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 5:219–234 [PubMed: 16518375]
- Aleman C, Annereau JP, Liang XJ et al. (2003) P-glycoprotein, expressed in multidrug resistant cells, is not responsible for alterations in membrane fluidity or membrane potential. *Cancer Res* 63:3084–3091 [PubMed: 12810633]
- Broxterman HJ, Lankelma J, Pinedo HM et al. (1997) Theoretical and practical considerations for the measurement of P-glycoprotein function in acute myeloid leukemia. *Leukemia* 11:1110–1118 [PubMed: 9204999]
- Broxterman HJ, Lankelma J, Pinedo HM (1996) How to probe clinical tumour samples for P-glycoprotein and multidrug resistance-associated protein. *Eur J Cancer* 32A:1024–1033 [PubMed: 8763344]
- Pallis M, Das-Gupta E (2005) Flow cytometric measurement of functional and phenotypic P-glycoprotein. *Methods Mol Med* 111:167–181 [PubMed: 15911979]
- Leith CP, Kopecky KJ, Chen IM et al. (1999) Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. *Blood* 94:1086–1099 [PubMed: 10419902]
- Krishan A (2000) Monitoring of cellular resistance to cancer chemotherapy: drug retention and efflux In: Darzynkiewicz Z, Crissman HA, Robinson JP (eds) *Cytometry*, part B, 3rd edn. Academic, San Diego, CA, pp 193–209
- Krishan A (2002) Flow cytometric monitoring of drug resistance in human tumor cells. *Methods Cell Sci* 24:55–60 [PubMed: 12815291]
- Ford J, Hoggard PG, Owen A, Khoo SH, Back DJ (2003) A simplified approach to determining P-glycoprotein expression in peripheral blood mononuclear cell subsets. *J Immunol Methods* 274:129–137 [PubMed: 12609539]
- Feller N, Kuiper CM, Lankelma J et al. (1995) Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* 72:543–549 [PubMed: 7669559]
- Aszalos A, Weaver JL (1998) Estimation of drug resistance by flow cytometry In: Jaroszeski MJ, Heller R (eds) *Flow cytometry protocols*. Humana, Totowa, NJ, pp 117–122
- Beck WT, Grogan TM, Willman CL et al. (1996) Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Res* 56:3010–3020 [PubMed: 8674056]

14. Wang EJ, Casciano CN, Clement RP, Johnson (2000) In vitro flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. *Drug Metab Dispos* 28:522–528 [PubMed: 10772630]
15. Janneh O, Jones E, Chandler B, Owen A, Khoo SH (2007) Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4 T cells and primary human lymphocytes. *J Antimicrob Chemother* 60:987–993 [PubMed: 17890284]
16. Meaden ER, Hoggard PG, Maher B, Khoo SH, Back DJ (2001) Expression of P-glycoprotein and multidrug resistance-associated protein in healthy volunteers and HIV-infected patients. *AIDS Res Hum Retroviruses* 17:1329–1332 [PubMed: 11602043]
17. Braga F, Ayres-Saraiva D, Gattass CR, Capella MA (2007) Oleanolic acid inhibits the activity of the multidrug resistance protein ABCB1 (MRP1) but not of the ABCB1 (P-glycoprotein): possible use in cancer chemotherapy. *Cancer Lett* 248:147–152 [PubMed: 16889888]
18. Echevarria-Lima J, Kyle-Cezar F, DF PL, Capella L, Capella MA, Rumjanek VM (2005) Expression and activity of multidrug resistance protein 1 in a murine thymoma cell line. *Immunology* 114:468–475 [PubMed: 15804283]
19. McAleer MA, Breen MA, White NL, Matthews N (1999) pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *J Biol Chem* 274:23541–23548 [PubMed: 10438534]
20. Liedert B, Materna V, Schadendorf D, Thomale J, Lage H (2003) Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin. *J Invest Dermatol* 121:172–176 [PubMed: 12839578]
21. Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH (2001) Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. *Clin Cancer Res* 7:1798–1804 [PubMed: 11410522]
22. Belinsky MG, Guo P, Lee K et al. (2007) Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. *Cancer Res* 67:262–268 [PubMed: 17210706]
23. Doyle LA, Ross DD (2003) Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* 22:7340–7358 [PubMed: 14576842]
24. Minderman H, Suvannasankha A, O'Loughlin KL et al. (2002) Flow cytometric analysis of breast cancer resistance protein expression and function. *Cytometry* 48:59–65 [PubMed: 12116365]
25. Kawabata S, Oka M, Soda H et al. (2003) Expression and functional analyses of breast cancer resistance protein in lung cancer. *Clin Cancer Res* 9:3052–3057 [PubMed: 12912956]
26. Ee PL, He X, Ross DD, Beck WT (2004) Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference. *Mol Cancer Ther* 3:1577–1583 [PubMed: 15634651]
27. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM (2000) Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 60:47–50 [PubMed: 10646850]
28. Scharenberg CW, Harkey MA, Torok-Storb B (2002) The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferential expressed by immature human hematopoietic progenitors. *Blood* 99:507–512 [PubMed: 11781231]
29. Zamber CP, Lamba JK, Yasuda K et al. (2003) Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics* 13:19–28 [PubMed: 12544509]
30. Vethanayagam RR, Wang H, Gupta A et al. (2005) Functional analysis of the human variants of breast cancer resistance protein: I206L, N590Y, and D620N. *Drug Metab Dispos* 33:697–705 [PubMed: 15743976]
31. Doyle LA, Yang W, Gao Y, Ordonez JV, Ross DD (1996) Novobiocin increases the accumulation of daunorubicin in an atypical multidrug-resistant breast cancer subline. *Proc Am Soc Clin Oncol* 15:398

32. Hausner P, Venzon DJ, Grogan L, Kirsch IR (1999) The “comparative growth assay”: examining the interplay of anti-cancer agents with cells carrying single gene alterations. *Neoplasia* 1:356–367 [PubMed: 10935491]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 7.1.

Primary structure of P-glycoprotein as positioned in cell membrane, with three antibodies (MRK16, UIC-2, and 17F9) positioned at the known binding epitopes (Adapted from Ambudkar et al. (2003) P-glycoprotein: from genomics to mechanism. *Oncogene* 22:7468–7485, with permission of the author).

Fig. 7.2.

Differences in histogram intensity after binding anti-mouse FITC, MRK16-FITC, or MRK-16 + anti-mouse FITC to P-glycoprotein-expressing NIH3T3MDR cells (From Aszalos and Weaver (1998) Estimation of drug resistance by flow cytometry. In: Jaroszeski and Heller (eds) Flow cytometry protocols. Humana, Totowa, NJ, pp 117–122, Figure 2, p. 121, with kind permission of Springer Science).

Fig. 7.3.

Influence of inhibitors on the side population of A549 cells stained with Hoechst 33342 dye (From Scharenberg et al. (2002) The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 99:507–512, used by permission).

Table 7.1

Flow cytometers and characteristic substrates used for detection of P-glycoprotein

Cytometer	Laser	Detectors and filters				References
		FL1	FL2	FL3	FL4	
FACSCan	488	530	585	650	NA	(5, 7)
FACSCalibur	488	530	585	670	NA	(3, 14)
Epics-XL	488	525	575	620	670	(10)
		FL1	FL2		FL3	FL4
Accumulation and efflux		Rhodamine 123 DiOC ₂ (3) Calcein-AM	Doxorubicin Daunorubicin		–	–
Fluorochromes for antibodies		FITC	PE		PE-TR ^a	–
Viability		–	PI		7-AAD ^b	7-AAD ^c

FITC fluorescein, *PE* phycoerythrin, *PE-TR* PE-Texas red tandem, *PI* propidium iodide, *7-AAD* 7-aminoactinomycin D

^aPE-Texas Red (PE-TR) emission is detected suboptimally in the 650 and 670 filters. More suitable fluorochromes may be PerCP and the PE tandem dyes PE-Cy5, PE-Cy5.5, or PE-Cy7

^bFACScan and FACSCalibur

^cEpics-XL

Table 7.2

Flow cytometers and characteristic substrates used for detection of multidrug resistance-associated protein

Cytometer	Laser	Detectors and filters				References
		FL1	FL2	FL3	FL4	
FACSCan	488	530	585	650	NA	(11, 19)
FACSCalibur	488	530	585	670	NA	(17)
Epics-XL	488	525	575	620	670	(15)
		FL1	FL2	FL3	FL4	
Accumulation and efflux		CMFDA CFDA FDA BCECF-AM Calcein-AM	Doxorubicin Daunorubicin TMR	–	–	
Fluorochromes for antibodies		FITC	PE	–	–	

FITC fluorescein, *PE* phycoerythrin, *CMFDA* 5-chloromethyl fluorescein diacetate, *CFDA* carboxy fluorescein diacetate, *FDA* fluorescein diacetate, *BCECF-AM* 2',7'-bis-(2carboxyethyl)-5-(and 6-)carboxy fluorescein acetoxymethyl ester, *TMR* tetra methyl rosamine

Table 7.3

Flow cytometers and characteristic substrates used for detection of breast cancer resistance protein

Cytometer	Laser	Detectors and filters				References
		FL1	FL2	FL3	FL4	
FACSCan	488	530	585	650	NA	(24, 25)
FACSCalibur	488, 635	530	585	670	660	(26, 27, 30)
Epics-XL	488	525	575	620	670	(10)
	FL1	FL2	FL3	FL4		
Accumulation and efflux	Topotecan BODIPY-prazosin Pheophorbide a BBR3390 Rhodamine 123 ^c DiOC ₂ (3) ^c Calcein-AM ^c	Topotecan Doxorubicin Daunorubicin	Mitoxantrone ^a	Mitoxantrone ^b		
Fluorochromes for antibodies	FITC GFP	PE	–	–		
Viability	–	PI	–	–		

FITC fluorescein, *PE* phycoerythrin, *PI* propidium iodide, *GFP* green fluorescent protein

^aFACSCalibur and Epics-XL, 488 laser, emission at 670 (Calibur) or 620 (Epics)

^bFACSCalibur, 635-nm laser, emission at 660

^cThese substrates are used to exclude Pgp and MRP1

Table 7.4

Side population cells for detection of breast cancer resistance protein

Cytometer	Lasers	Detectors, emission filters, and dichroic mirrors					References
		UV–blue	UV–red	Dichroic mirror	PI ^a	7-AAD ^a	
FACS Vantage	488, 355	424/44	675LP	640LP	585	650	(28)
Mo-Flo LSRII	488, 355 488, 355	For these cytometers, combinations of parameters as given below in footnote b can be used ^b					

^aThe viability dyes propidium iodide or 7-aminoactinomycin D may be used; both are excited at 488 nm

^bA number of filter combinations have been used for the measurement of the blue and red emissions of the Hoechst 33342 dye, which is excited at 351–364 nm. These include 440/40, 450/50, and 450/20 bandpass filters for the blue, 670LP for the red, and 600LP, 610LP, or 635LP for the dichroic mirror