11

Flow Cytometric Monitoring of Fluorescent Drug Retention and Efflux

Awtar Krishan and Ronald M. Hamelik

Summary

Laser flow cytometry has been used for monitoring cellular retention of fluorescent drugs such as fluorescent anticancer antibiotics (e.g., doxorubicin) and fluorochromes used for the detection of cellular drug efflux and resistance (e.g., rhodamine 123, Hoechst 33342). Multiparametric flow cytometry can be used for identification of tumor cell subpopulations based on their drug retention profiles with or without the presence of an efflux blocker. This rapid procedure can be used for identification of tumor cells with the drug-resistance phenotype based on drug efflux as well as for efflux blockers that may block efflux of a chemotherapeutic agent and thus increase cellular retention and sensitivity. It has been reported recently that some of the bone marrow stem cells (SP cells) efflux the Hoechst 33342 fluorochrome and thus can be rapidly identified by comparing red vs blue fluorescence in the presence or absence of an efflux blocker such as verapamil. The present chapter discusses some of the flow cytometric methods used for the study of cellular drug retention and the artifacts that may arise in such analysis.

Key Words

Drug efflux; drug fluorescence; drug resistance; drug retention; drug transport; flow cytometry; multiple drug resistance (MDR).

1. Introduction

Flow cytometric monitoring of drug retention and efflux is a useful technique for the study of drug resistance. Most published work has focused on laser excitation of cellular fluorescent drug content and its modulation by efflux blockers. In multiparametric flow analysis, data on drug fluorescence can be correlated with other parameters such as cell size or expression of different cellular markers. Two major uses of this methodology have been for identification of agents that block drug efflux and increase intracellular drug retention and for the rapid identification of cells that have drug efflux as a phenotypic characteristic. A

From: *Methods in Molecular Medicine, vol. 111: Chemosensitivity: Vol. 2: In Vivo Models, Imaging, and Molecular Regulators*
Edited by: R. D. Blumenthal © Humana Press Inc., Totowa, NJ

more recent and important use of flow cytometry has been for the rapid identification of a subset of stem cells (SP) characterized by reduced retention and efflux of Hoechst 33342 fluorochrome ***(1,2)***.

Drug resistance due to reduced drug influx and/or enhanced efflux may be a major cause for failure of chemotherapy in refractory cancer patients. Starting with the pioneering work of Ling, Biedler, Dano, Kessel, and their colleagues ***(3–6)*** on cell lines made resistant in vitro by exposure to increasing concentrations of colchicine, actinomycin-D, or adriamycin, multiple drug resistance (MDR) was identified as a major target for clinical protocols seeking to overcome the efflux pump and enhance drug retention and sensitivity. The genetic basis and the proteins responsible for drug efflux have been identified ***(7)***, and several protocols and drugs were tested to overcome the efflux pump ***(8)***. One of the major lessons learned during the previous decade is that drug resistance is multifactorial and the drug-resistant cells use a variety of protective mechanisms to reduce cellular damage.

Several proteins that have the unique ability to act as efflux pumps have been identified. Most of the earlier work focused on MDR gene that codes for the cell membrane-resident P-glycoprotein (P-gp). Subsequent studies have identified two other proteins, MRP and LRP, that reduce cellular drug retention and chemosensitivity ***(9,10)***. With the availability of antibodies against the P-glycoproteins, immunocytochemistry was the primary tool for monitoring the expression of these proteins. Subsequently, laser flow cytometry was used to study transport and subcellular distribution of fluorescent drugs ***(11,12)***. Because cellular retention and distribution is related to cytotoxicity, these "functional studies" have the potential to supplement the immunocytochemical data and report on the functional activity of the efflux proteins ***(13–15)***.

To quantitate markers and mechanisms involved in cellular resistance requires knowledge about a variety of sophisticated laboratory techniques involving immunocytochemistry, flow cytometry, and molecular biology. Unlike immunocytochemistry where one can identify cells with positive or negative P-glycoprotein expression, laser flow cytometry can rapidly determine cellular fluorescent drug retention in the presence or absence of an efflux blocker. However, in spite of its sensitivity, convenience, and rapidity, the use of flow cytometry for functional analysis of drug retention and the effect of efflux blockers can be fraught with the danger that artifacts may lead to erroneous conclusions. We have used a panel of well-characterized cell lines, fluorochromes, and efflux blockers to identify some of the problems in the use of flow cytometry for drug resistance-related marker expression and functional assays ***(11–16)***.

Laser flow cytometry can be used for the monitoring of the following parameters related to drug transport and resistance: (1) cellular transport of fluorescent drugs (e.g., anthracyclins, rhodamine 123, Indo-1 AM, Calcein, Hoechst

33342); (2) effect of drugs that either increase drug influx (e.g., amphotericin B) or enhance drug retention by blocking efflux (e.g., verapamil, phenothiazines, cyclosporins, tamoxifen); (3) effect of efflux blocker combinations on drug retention; (4) heterogeneity in drug retention and response to efflux blockers of subpopulations in a tumor; (5) selection of effective efflux blockers and protocols for possible clinical use; (6) rapid identification and sorting of cells (e.g., SP cells) that have efflux as a major phenotypic marker.

2. Materials

2.1. Indicator Cell Lines

In performing flow cytometric studies on drug transport and efflux, it is important that the procedures used for specimen preparation and analysis are proper to yield the expected results. This makes the use of well-known human or rodent indicator cell lines as controls and calibrators important. Several paired human and mouse parental (drug-sensitive and -resistant) cell lines are available for this purpose. We have used the mouse leukemic P388 cell line and it's cloned adriamycin-resistant P388/R84 cell line for most of our work ***(16)***. Another adriamycin (doxorubicin)-resistant cell line, P388/ADR, is widely available from various sources ***(6,17)***. These lines are easy to maintain as suspension cultures or in vivo as ascites and have relatively short doubling times (16–18 h). The P388/R84 cell line has multifactorial drug resistance involving drug efflux, detoxification mechanism, and DNA damage/repair ***(16)***. It also has relatively stable drug resistance and does not need frequent doxorubicin exposure to maintain the resistant phenotype. Several other well-characterized paired human cell lines useful for the study of drug retention and efflux include human lymphoid cell line, CCRF-CEM, and its vinblastine-resistant subline, CCRF-CEM$_{VLB100}$, developed and studied extensively by Beck and his colleagues ***(18)***. Though the parental CCRF-CEM cell line was originally diploid, several tetraploid drug-resistant CCRF-CEM cell lines are available and are easy to maintain in suspension cultures. The human colon carcinoma cell lines, SW620 and its adriamycin-resistant subline, SW620/AD300, are also good paired cell lines to maintain in the lab ***(19)***. In the resistant cell line, drug efflux is possibly the major and only mechanism responsible for resistance. The SW620 cell lines grow as monolayers, double in 24 h, and can also be grown as xenografts in athymic mice. However, most of these resistant cell lines need to be continuously grown or frequently rechallenged with drugs to maintain their efflux mechanism.

2.2. Fluorochromes

For flow cytometric studies on drug retention and efflux, the dyes chosen must be excitable by a high-pressure mercury arc lamp or emission from a spe-

cific laser line. The most commonly used fluorochromes for drug transport studies are doxorubicin (Adriamycin, NSC-123127, Adria Labs, Columbus, OH), daunorubicin, NSC-821151, Calbiochem, San Diego, CA), and rhodamine 123 (Calbiochem). These fluorochromes can be excited from the 488-nm argon laser line. The Hoechst 33342 (Calbiochem) dye is excitable with the mercury arc lamp or the UV laser lines from an argon laser. Bodipy-verapamil (Molecular Probes, Eugene, OR) and the calcium indicator dye Indo-1 AM (Molecular Probes) are two other fluorochromes used for the study of drug transport.

Much of the early drug efflux functional assays relied on the use of important chemotherapeutic antibiotics, Doxorubicin or daunorubicin, for the measurement of drug retention. Daunorubicin is lipophilic and rapidly transported into cells in 15–30 min. The uptake of doxorubicin is much slower and takes 2–3 h to reach maximum. These antibiotics are excited by the standard 488-nm argon laser line and quench their fluorescence on binding to DNA ***(11)***. Rhodamine 123 is rapidly transported and its fluorescence, mostly located in the mitochondria, is much brighter than that of daunorubicin or doxorubicin ***(21)*** (*see* **Note 1**). We have earlier compared the use of two new fluorochromes, SY-38 and SY-3150 ***(20)*** for monitoring of drug efflux. Different fluorochromes used for transport studies differ in their chemical binding characteristics as well as transport and retention properties. We ***(20)*** have reported that cellular drug retention data cannot be extrapolated between different fluorochromes, and tumor cells should be tested for their transport characteristics and sensitivity to efflux blockers with drug that will be used for treatment of the patient ***(15)***.

The histograms in **Fig. 1** are of normal human peripheral blood lymphocytes, isolated on a Ficoll-Hypaque gradient, incubated with four different fluorochromes: daunorubicin (DNR), rhodamine 123 (RH-123), SY-38 (SY38), and SY-3150 (SY3150). Cells incubated with DNR **(Fig. 1A)** show a single predominant population, while cells incubated with RH-123 **(Fig. 1B)** show a distinct second population (arrow). Similarly, cells incubated with SY38 also showed a second distinct population (arrow). Cells incubated with SY3150 **(Fig. 1D)** did not show any distinct extra populations like those seen in **Fig. 1B** and **Fig. 1C**. The conclusion to be drawn is that the degree of heterogeneity seen depends on the fluorochrome used. In this example, daunorubicin, rhodamine 123, and SY-3150 are not as good as SY-38 for measuring drug transport and efflux.

Other fluorochromes that can be used for study of drug transport are discussed in the Molecular Probes Handbook of Fluorescent Probes and Research Chemicals section on Probes for Cell Adhesion, Chemotaxis, Multidrug Resistance and Glutathione, which the reader may find at www.probes.com/handbook/sections/1506.html.

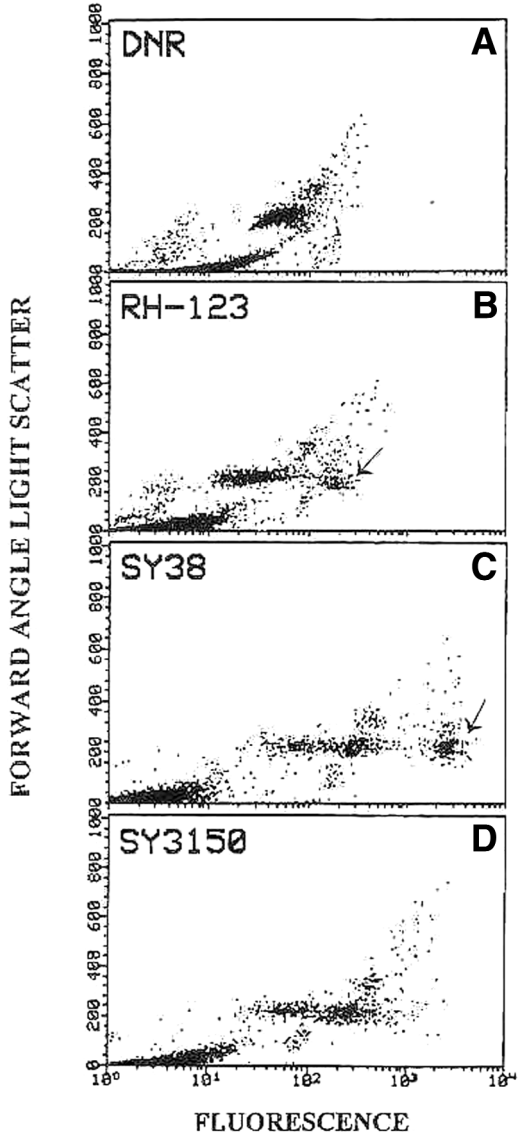

Fig. 1. Dot plots of forward scatter vs drug fluorescence of human peripheral blood lymphocytes incubated with four different fluorochromes **(A–D)**. The difference in heterogeneity displayed shows the importance of choosing the best fluorochrome for the cells being studied. (Adapted from **ref. *20***.)

2.3. Drug Efflux Blockers

Several well-known drugs with a wide variety of clinical uses have been shown to modulate drug retention. Well-known efflux blockers include calcium channel blockers (e.g., verapamil), immunosuppressive drugs (e.g., cyclosporins), platelet-active agents (e.g., dipyridamole), psychosomatic drugs (e.g., phenothiazines), antimalarials (e.g., quinine), and antiestrogens (e.g., tamoxifen) ***(22–24)***. Stein et al. ***(25)*** have shown that a combination of efflux blockers may have synergistic effect on blocking the pump mechanism (*see* **Note 2**).

3. Methods

Protocols for monitoring of drug retention and efflux need to have a control sample, preferably paired cell lines of drug-sensitive and -resistant phenotype; a fluorochrome of interest; a known efflux blocker that will enhance retention of the fluorochrome; and a data acquisition protocol for the flow cytometer.

3.1. Control Cell Lines

For calibration and controls, we routinely use log-phase suspension cultures of doxorubicin-resistant P388/R84 cells ***(16)***, grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin. The ID50 for the parental P388 and P388/R84 cells in soft agar assays are 0.0875 and 8.4 μ*M* of doxorubicin, respectively. Cells (10^6/mL) are incubated with doxorubicin (1 μ*M*, 1 h), daunorubicin (0.1 μ*M*, 30 min), or rhodamine 123 (0.1 μ*M*, 15 min) at 37°C. A second aliquot of cells is incubated with the fluorochrome and efflux blocker (verapamil, 10 μ*M*), prochlorperazine (15 μ*M*), or dipyridamole (10 μ*M*). Cells incubated in the presence of the efflux blocker are analyzed for their fluorescence and the laser power and gain/amplification adjusted to record the peak of fluorescence distribution in the second log of a four-log histogram. In resistant cells incubated without the efflux blocker, there is approx one log less fluorescence. Once the laser power, photomultiplier high voltage, and other gains have been optimized (using the resistant cells with and without the efflux blocker), the settings are not changed for running the experimental samples.

3.1.1. Experimental Sample

Cells are recovered by centrifugation of human body fluids (pleural fluid, bone marrow, peripheral blood, ascites fluid) or of solid tumor homogenates dissociated by enzymatic or mechanical means. The supernatant fluid is aspirated and the cell pellet is resuspended and washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). After centrifugation, the pelleted cells are resuspended in fresh tissue culture medium supplemented with 10% heat-inactivated FBS and filtered through a 40-μ*M* nylon mesh.

Bone marrow aspirates, peripheral blood, and some body fluid samples may contain large numbers of red blood cells, which need to be removed. These cell pellets are diluted with Ca^{2+}- and Mg^{2+}-free PBS, layered over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged. The mononuclear cell layer is carefully aspirated, washed with Ca^{2+}- and Mg^{2+}-free PBS, and resuspended in fresh tissue culture medium supplemented with 10% heat-inactivated FBS.

3.2. Fluorochromes

Stock solutions of doxorubicin, daunorubicin, or rhodamine 123 are prepared in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS), and fresh working solutions are prepared before each experiment. The final drug concentrations normally used is 1–3 μ*M* doxorubicin or daunorubicin or 0.1 μ*M* rhodamine 123, and cells may be incubated for 15–60 min at 37°C before flow analysis (*see* **Note 3**).

3.3. Efflux Blockers

The blockers we normally use are prochlorperazine, verapamil, or dipyridamole. The stock solutions are prepared in Ca^{2+}- and Mg^{2+}-free HBSS, and fresh working solutions are prepared before each experiment (*see* **Note 3**). For efflux-blocking experiments, cells incubated with a fluorochrome are incubated with and without the addition of prochlorperazine (20 μ*M*), verapamil (10 μ*M*), or dipyridamole (15 μ*M*) (*see* **Note 4**).

3.3.1. Data Acquisition Protocols

Although our published work on drug retention and efflux has been carried out on a Beckman Coulter XL (Beckman Coulter, Miami, FL) or a Becton Dickinson FACScan (San Jose, CA) equipped with an argon-ion laser, most of the currently available flow cytometers including those with high-pressure mercury illumination have enough excitation power for performing the drug uptake and efflux experiments (*see* **Note 5**). In most studies, forward angle (FS), 90° light scatter (SS), and fluorescence >530 nm (FL1) are measured and the same flow cytometer data acquisition protocol can be used for measuring drug uptake/efflux, efflux blocking, and the separation of live/dead cells with propidium iodide (PI). The flow cytometer desktop is arranged to collect three sets of histograms/scatter plots:

1. A two-parameter FS Y-axis vs SS X-axis histogram for setting the FS gain and visualizing live/dead/debris. Set the gain so that the populations are about mid-scale and high enough to visually separate cells from debris.

2. A one-parameter log FL1 histogram with the high voltage (HV) set to record fluorescence of the resistant cells incubated with the fluorochrome and the efflux blocker in the second log of a four-log-scale X axis.
3. A two-parameter FS Y-axis vs FL1 X-axis histogram for visualizing drug-sensitive/resistant cells, the effect of blocked/nonblocked cells, or live/dead shift with PI.

Minimums of 10,000 cells are usually analyzed to generate list-mode data files (*see* **Note 6**). In kinetic studies of drug uptake, "time" is used as a parameter along with drug fluorescence. The data acquisition software on most of the commercial flow cytometers has "time" as a parameter. The data acquisition protocol for kinetic drug uptake studies has an additional two-parameter histogram of FL1 Y axis vs time X axis. As time is used as the stopping parameter for data acquisition, more than 10,000 cells may be analyzed in a typical kinetic study, depending on the event rate and cell concentration.

3.3.2. Types of Assays

1. For drug uptake/efflux studies, cells are incubated with the fluorochrome with or without the efflux blocker for a given length of time and then analyzed on the flow cytometer. The appearance of subpopulations, which increase their fluorochrome retention in the presence of the efflux blockers, suggests the presence of an active drug efflux pump.

   The multiparameter histograms in **Fig. 2** are of P388 doxorubicin-sensitive **(2A)** and -resistant **(2C)** cells, respectively, incubated for 30 min with 2 µmol of daunorubicin. **Figures 2B** and **2D** are with daunorubicin and 10 µmol of verapamil. The drug fluorescence (X axis) in log scale shows that the resistant cells **(2C)** have a four- to sixfold reduced drug retention vs the drug-sensitive cells. That this reduced retention is due to rapid drug efflux is shown by comparison of **Fig. 2C** and **2D**, where verapamil has blocked the drug efflux pump in the resistant cells, resulting in similar drug retention of the sensitive and the resistant cells.
2. For drug-uptake kinetic studies, the fluorochrome and either PBS or the efflux blocker is added to the cells at time zero (T_0) and data acquisition started. The shift in fluorescence intensity vs time is a measure of drug retention, which in resistant cells incubated in the presence of the efflux blockers is significantly enhanced. For these studies it is suggested that the event rate of approx 100 events/s be maintained. As drug uptake is influenced by temperature, it is important to keep the sample tube at 37°C during the entire data acquisition run. This can be achieved by use of a temperature-controlled heated air curtain. Scale the time X axis to suit the known drug uptake characteristics of the fluorochrome. For example, rhodamine 123 and daunorubicin can be studied using a 600-s (10 min) scale, whereas doxorubicin needs 30–60 min to study its transport.

   The contour plots in **Fig. 3** show log fluorescence vs time of P388/R84 cells incubated for 10 min with rhodamine 123 with or without verapamil (top plot). In less than a minute, the drug efflux pump was blocked; and after 10 min of incu-

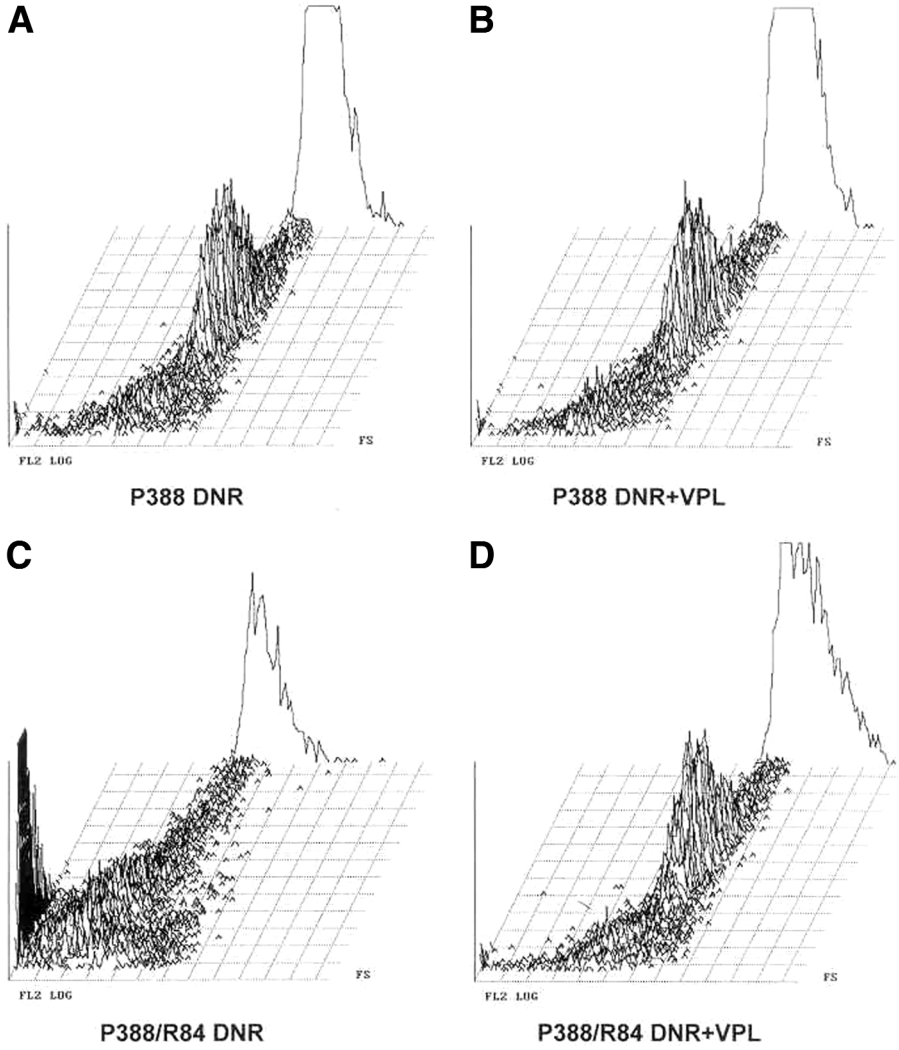

Fig. 2. Uptake and rentention of Daunorubicin in P388 parental drug-sensitive and P388/R84 drug-resistant cells. In P388 cells **(A,B)** and in P388/R84 cells incubated with the efflux blocker verapamil **(D)** a prominent population with high drug retention is seen. In P388/R84 cells incubated with daunorubicin alone, retention is low due to drug efflux **(C)**. Adapted from **ref. *30***.

bation, these cells had approximately log higher fluorescence intensity than the control cells.

3. For identification of dead or membrane-permeable cells, it is essential to discriminate between the high fluorescence of these cells and that of the cells that

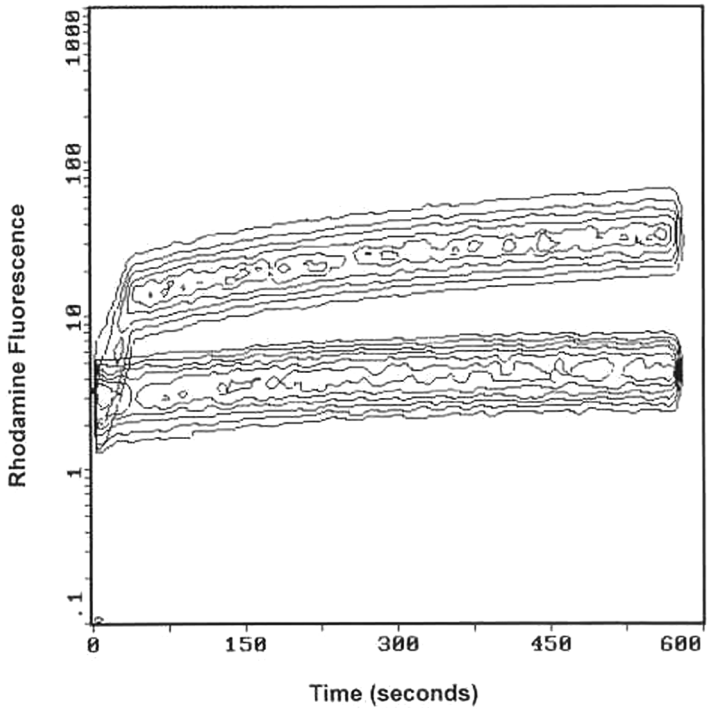

Fig. 3. Time vs fluorescence of rhodamine 123 in P388/R84 cells incubated with drug alone (lower contour plot) or in the presence of the efflux blocker verapamil (upper contour plot). (Adapted from **ref. *30***.)

lack the drug efflux pump. We have described the inclusion of isotonic propidium iodide for staining of dead or membrane-damaged cells in drug kinetic and efflux studies ***(20)***. We recommend addition of 25 µg/mL of propidium iodide dissolved in isotonic phosphate buffered saline for this purpose. **Figures 4A** and **4E** are dot plots of murine leukemic P388 and drug-resistant P388/R84 cells incubated with daunorubicin. Based on the forward scatter-vs-log fluorescence dot plots **(Fig. 4E)**, the P388/R84 culture had at least five distinct subpopulations. The single-parameter histograms of drug fluorescence (**Figs. 4B** and **4F**), do not demonstrate heterogeneity present in the sample. To discriminate among the cells with low drug retention (resistant cells), cells with high retention (sensitive cells), and cells with damaged cell membranes (which cannot efflux the drug), isotonic propidium iodide was added to the cultures to label the membrane permeable cells. The dot plot **(Fig. 4C)** and single-parameter histogram **(Fig. 4D)** show how the procedure can enhance the separation of the subpopulations by dramatically increasing the fluorescence of the membrane-permeable cells. This separation is even more pronounced in the P388/R84 cells, as the live resistant cells efflux the fluorochrome while the membrane-permeable cells (**Figs. 4G** and **4H**) stain brightly with PI

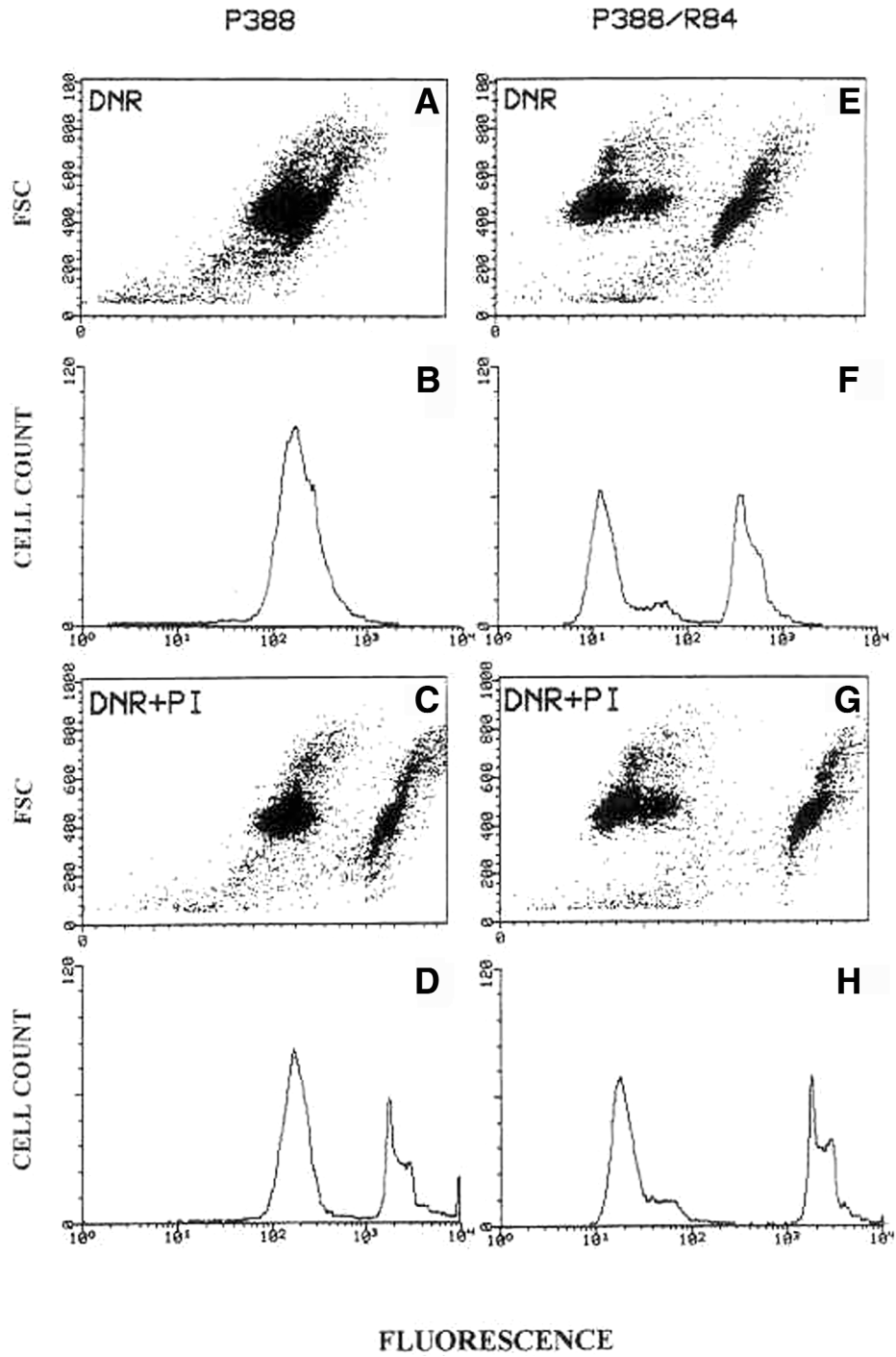

Fig. 4. Use of isotonic propidium iodide for identification of cells with permeable cell membranes or dead cells. (Adapted from **ref.** ***20***.)

and stand out as distinct populations. This experiment shows that by using PI, one can distinguish between the real heterogeneity of drug retention in a tumor specimen and the artifacts created by the presence of dead, dying, and necrotic cells with leaky dye-permeable membranes.

Drug efflux and sensitivity to efflux blockers is not limited to drug-resistant tumor cells, as P-glycoprotein expression and drug efflux has been reported in a variety of normal cells such as capillary endothelial cells of the brain and testes, adrenal, liver, and CD34-positive bone marrow stem cells. Although once reported to have no P-gp mRNA expression, a significant percentage of normal human peripheral lymphocytes have P-gp expression and efflux that can be blocked. The histograms in **Fig. 5** are of normal human peripheral blood lymphocytes, isolated on a Ficoll-Hypaque gradient, incubated with the fluorochrome SY-38 and the efflux blockers verapamil (Vpl) or dipyridamole (Dpd). **Figure 5A** is a forward-scatter-vs-side-scatter dot plot of these isolated mononuclear cells. The R1 gate identifies small lymphocytes. The forward-scatter-vs-log fluorescence of all the mononuclear cells after incubation with SY-38 is shown in **Fig. 5B** and in the presence of SY-38 and verapamil in **Fig. 5C**. In comparing these two images, it appears that cells in **Fig. 5B** (arrows) have increased their drug retention in the presence of verapamil **(Fig. 5C)**. **Figures 5D** and **5E** show the data using the R1 gate region for small lymphocytes, after incubation with SY-38 alone **(Fig. 5D)** or in the presence of verapamil **(Fig. 5E)**. This data shows that cells in the R1 gate, which had low SY-38 retention, were sensitive to the efflux blocking action of verapamil or other efflux blockers (dipyridamole, data not shown).

Figure 6 illustrates daunorubicin retention in tumor cells from pleural effusion of a lung cancer patient after incubation with and without the efflux blockers chlorpromazine (CpZ) or verapamil (VpL). **Figure 6A** shows heterogeneity of daunorubicin retention. **Figure 6B** shows that chloropromazine blocked the efflux pumps, producing a single fluorescence peak, while verapamil did not **(Fig. 6C)**. Histograms **6G–I** shows similar analysis of cells retrieved from the patient after 1 mo of therapy ***(15)***.

4. For flow cytometric identification of SP stem cells in bone marrow, Goodell et al. ***(1)*** reported that in murine bone marrow cells incubated with Hoechst 33342 for red vs blue emission, a small subpopulation of cells (SP cells) with reduced drug fluorescence could be recognized. These SP cells appear to have active drug efflux that was blocked by incubation with verapamil, resulting in increased fluorescence. The Hoechst staining method based on drug efflux and blocking is described as a simple, easy, and reproducible way for identification of hematopoietic stem cells. In subsequent dual-wavelength analysis of Hoechst dye-stained human, rhesus and miniature swine bone marrow cells, SP-like cells, were identified as a distinct population of cells that efflux the dye in a manner identical to that of the murine SP cells. Like the murine SP cells, both human and rhesus SP cells are primarily CD34-negative and lineage marker-negative. The rhesus SP population contains a large number of long-term culture-initiating cells (LTC-ICs), thus suggesting that they are primitive hematopoietic cells capable of

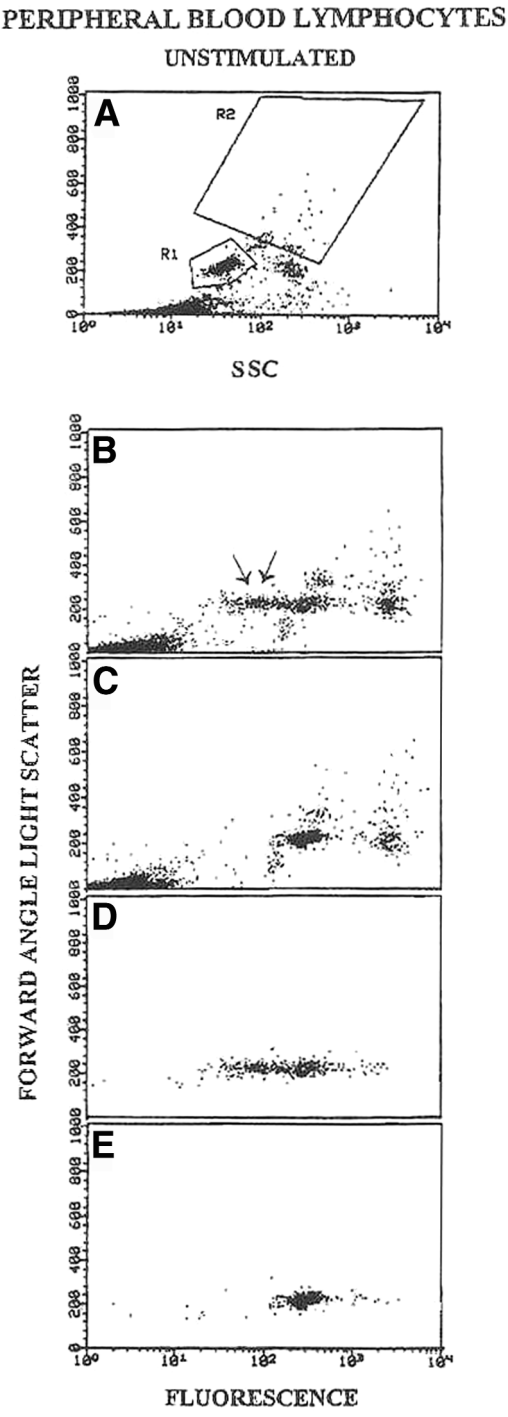

Fig. 5. Dot plots of unstimulated peripheral blood lymphocytes incubated with fluorochrome SY-38. (Adapted from **ref. *20***.)

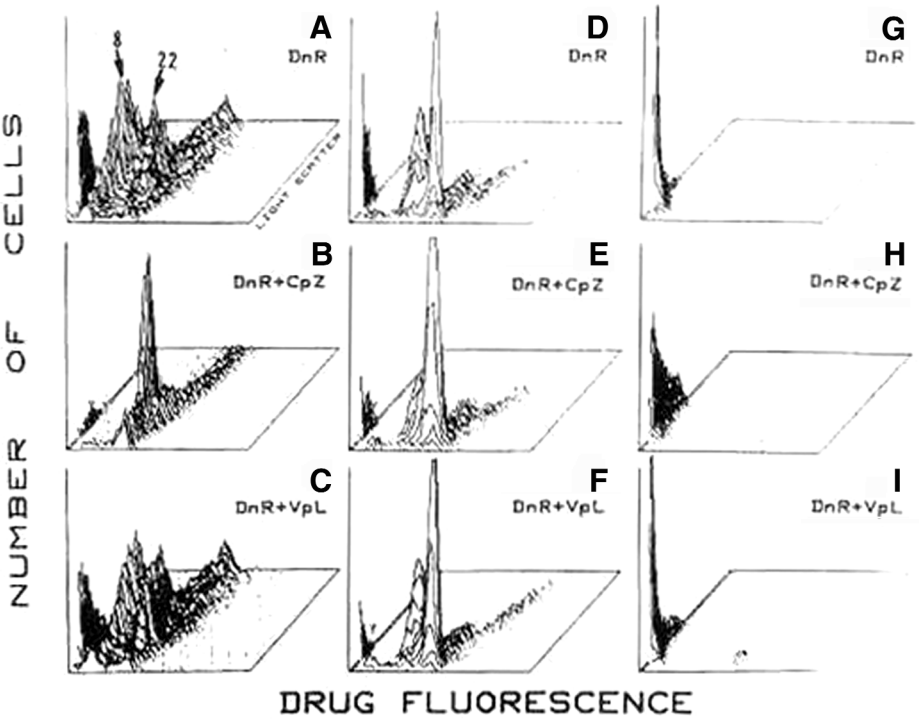

Fig. 6. Daunorubicin retention in tumor cells from the pleural fluid of a lung cancer patient. Note chlorpromazine CpZ **(B)** but not verapamil VpL **(C)** enhanced drug retention, resulting in the emergence of a single population with high drug fluorescence. In samples retrieved after 1 mo of therapy, drug retention was low **(G)**, and the efflux blockers did not increase drug retention **(H,I)**. (Adapted from **ref.** ***15***.)

differentiation into T-cells. In subsequent studies, the Hoechst double-emission method was used to describe the presence of SP cells in a variety of human tissues, including skeletal muscle, heart, brain liver, spleen, umbilical cord, and adult blood. Kim et al. have reported studies suggesting the involvement of the ABCG2 gene of the human ATP-binding cassette superfamily in Hoechst 33342 efflux in transformed MCF cell line *(27)*.

Figure 7 shows a red-vs-blue dot plot of Hoechst 33342 fluorescence in murine bone marrow cells analyzed on a Quanta™ Analyzer with HBO mercury light excitation (NPE Systems, Pembroke Pines, FL). The arrow in **Fig. 7A** points to the subpopulation (SP) of bone marrow cells with reduced Hoechst retention that are not seen in cells coincubated with the efflux blocker verapamil **(Fig. 7B)**.

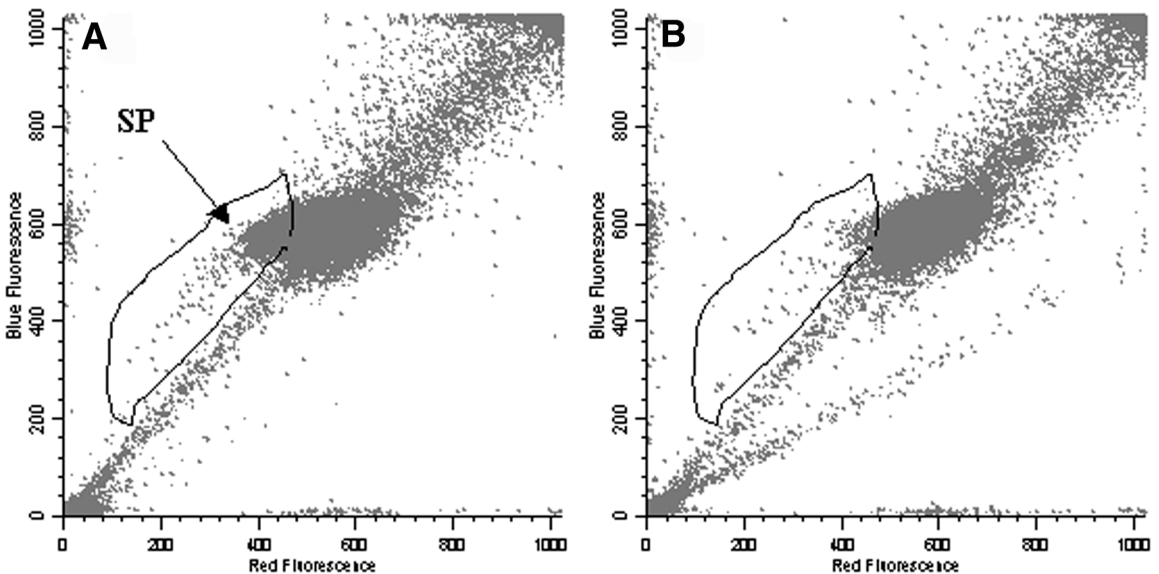

Fig. 7. Two color (red vs blue) emission dot plots of mouse bone marrow cells incubated with Hoechst 33342. In **(A)**, the arrow points to the presence of SP cells with low fluorochrome retention. In the presence of the efflux blocker verapamil **(B)**, drug retention in most of the SP cells is enhanced. (Data generated by Raquel Cabana on a NPE Quanta Analyzer [NPE Systems, Pembroke Pines, FL].)

4. Notes

1. Rhodamine 123 can exhibit a transient increase in binding to mitochondria of dead cells as well as in cells entering the cell cycle from a quiescent state, as reported by Darzynkiewicz et al. ***(28)***.
2. Very high concentrations of the fluorochromes or the efflux blockers may be toxic and damage the cell membrane and the efflux pump ***(26)***.
3. The pH of the incubating mixture sample is an important factor when considering drug transport and efflux studies, as the excitation maxima of a drug may be shifted or the uptake and efflux rates altered by pH changes ***(29)***.
4. Since some of the efflux blockers may bind to glass and rubber or precipitate out of solution under certain experimental conditions, proper precautions should be observed to avoid this artifact.
5. In flow cytometry, one must be sure that pinholes have not developed in aging dichroic filters or that autofluorescence is not generated by high-power excitation of the bandpass filters.
6. Dual-parameter dot plots or scattergrams of forward light scatter vs drug fluorescence are better than single-parameter histograms of cellular fluorescence, because they allow for identification of dead cells and other subpopulations for gating purposes.

References

1. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183,** 1797–1806.
2. Storms, R. W., Goodell, M. A., Fisher, A., Mulligan, R. C., and Smith, C. (2000) Hoechst dye efflux reveals a novel CD7(+)CD34(–) lymphoid progenitor in human umbilical cord blood. *Blood* **96,** 2125–2133.
3. Ling, V. (1992) P-glycoprotein and resistance to anticancer drugs. *Cancer* **69,** 2603–2609.
4. Biedler, J. L. (1994) Drug resistance: genotype versus phenotype. *Cancer Res.* **54,** 666–678.
5. Dano, K. (1973) Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochim Biophys. Acta* **323,** 466–483.
6. Kessel, D., Botterill, V., and Wodinsky, I. (1968) Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. *Cancer Res.* **28,** 938–941.
7. Gottesman, M. M. (1993) How cancer cells evade chemotherapy: Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res.* **53,** 747–754.
8. Gottesman, M. M. and Pastan, I. (1989) Clinical trials of agents that reverse multidrug-resistance. *J. Clin. Oncol.* **7,** 409–411.
9. Cole, S. P., Bhardwaj, G., Gerlach, J. H., et al. (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258,** 1650–1654.

10. Izquierdo, M. A., Scheffer, G. L., Flens, M. J., et al. (1996) Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. *Am. J. Pathol.* **148,** 877–887.
11. Krishan, A. and Ganapathi, R. (1980) Laser flow cytometric studies on the intracellular fluorescence of anthracyclines. Cancer Res. 40, 3895–3900.
12. Durand, R. E. and Olive, P. L. (1981) Flow cytometry studies of intracellular adriamycin in single cells in vitro. *Cancer Res.* **41,** 3489–3494.
13. Krishan, A. and Bourguignon, L.Y. (1984) Cell cycle related phenothiazine effects on adriamycin transport. *Cell. Biol. Int. Rep.* **8,** 449–456.
14. Krishan, A., Sauerteig, A., and Wellham, L. L. (1985) Flow cytometric studies on modulation of cellular adriamycin retention by phenothiazines. *Cancer Res.* **45,** 1046–1051.
15. Krishan, A., Sridhar, K. S., Davila, E., Vogel, C., and Sternheim, W. (1987) Patterns of anthracycline retention modulation in human tumor cells. *Cytometry* **8,** 306–314.
16. Nair, S., Singh, S. V., Samy, T. S., and Krishan, A. (1990) Anthracycline resistance in murine leukemic P388 cells. Role of drug efflux and glutathione related enzymes. *Biochem. Pharmacol.* **39,** 723–728.
17. Inaba, M., Kobayashi, H., Sakurai, Y., and Johnson, R. K. (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res.* **39,** 2200–2203.
18. Beck, W. T., Mueller, T. J., and Tanzer, L. R. (1979) Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. *Cancer Res.* **39,** 2070–2076.
19. Leibovitz, A., Stinson, J. C., McCombs, W. B. III, McCoy, C. E., Mazur, K. C., and Mabry, N. D. (1976) Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* **36,** 4562–4569.
20. Krishan, A., Sauerteig, A., Andritsch, I., and Wellham, L. (1997) Flow cytometric analysis of the multiple drug resistance phenotype. *Leukemia* **11,** 1138–1146.
21. Lampidis, T. J., Munck, J. N., Krishan, A., and Tapiero, H. (1985) Reversal of resistance to rhodamine 123 in adriamycin-resistant Friend leukemia cells. *Cancer Res.* **45,** 2626–2631.
22. Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.* **41,** 1967–1972.
23. Ganapathi, R., Grabowski, D., Rouse, W., and Riegler, F. (1984) Differential effect of the calmodulin inhibitor trifluoperazine on cellular accumulation, retention, and cytotoxicity of anthracyclines in doxorubicin (adriamycin)-resistant P388 mouse leukemia cells. *Cancer Res.* **44,** 5056–5061.
24. Slater, L. M., Sweet, P., Stupecky, M., Wetzel, M. W., and Gupta, S. (1986) Cyclosporin A corrects daunorubicin resistance in Ehrlich ascites carcinoma. *Br. J. Cancer* **54,** 235–238.
25. Stein, W. D. (1997) Kinetics of the multidrug transporter (P-glycoprotein) and its reversal. *Physiol. Rev.* **77,** 545–590.

26. Krishan, A., Sridhar, K. S., Mou, C., et al. (2000) Synergistic effect of prochlorperazine and dipyridamole on the cellular retention and cytotoxicity of doxorubicin. *Clin. Cancer Res.* **6,** 1508–1517.
27. Kim, M., Turnquist, H., Jackson, J., et al. (2002) The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. *Clin. Cancer Res.* **8,** 22–28.
28. Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J., and Melamed, M. R. (1982) Interaction of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.* **42,** 799–806.
29. Alabaster, O., Woods, T., Ortiz-Sanchez, V., and Jahangeer, S. (1989) Influence of microenvironmental pH on adriamycin resistance. *Cancer Res.* **49,** 5638–5643.
30. Krishan, A. (2001) Monitoring of cellular resistance to cancer chemotherapy: drug retention and efflux, in *Methods in Cell Biology, Vol 64* (Darzynkiewicz, Z., Crissman, H. A., and Robinson, J. P., eds.), Academic, San Diego, CA, pp. 193–209.

http://www.springer.com/978-1-58829-586-6

Chemosensitivity
Volume II: In Vivo Models, Imaging, and Molecular Regulators
Blumenthal, R.D. (Ed.)
2005, XVIII, 442 p., Hardcover
ISBN: 978-1-58829-586-6
A product of Humana Press