

Advances in Brief

Flow Cytometric Assay of Modulation of P-Glycoprotein Function in Whole Blood by the Multidrug Resistance Inhibitor GG918¹

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Abstract

We sought to develop an assay for measuring the inhibition of P-glycoprotein (Pgp) function in whole blood as an indicator of *in vivo* drug activity. Since the CD56⁺ subset of peripheral blood lymphocytes (PBLs) has been shown to express functional Pgp, the changes in rhodamine 123 (R123) uptake by CD56⁺ PBLs from GG918-treated and untreated whole blood were used as the basis for these studies. In an *ex vivo* study, heparin-treated whole blood was obtained from normal volunteers, and GG918 and R123 were added at various concentrations for time course analyses of dye loading. GG918 concentrations from 2.5 to 800 nM were tested in incubations ranging from 15 min to 3 h prior to R123 addition. R123 loading times ranged from 0 to 80 min. Flow cytometric analyses of the CD56⁺ PBLs indicated that the resolution of Pgp inhibition was dependent on inhibitor concentration and time of R123 loading and independent of the R123 concentrations tested. In this *ex vivo* assay model, a dose-dependent response was seen for GG918 with a 2-fold increase in cellular R123 intensity being produced at a drug concentration of 80 nM. When this assay method was applied to blood samples from volunteers dosed p.o. with GG918, similar shifts in R123 fluorescence of the CD56⁺ PBLs were observed with significant increases in R123 intensity occurring at serum concentrations as low as 40 nM. In contrast to assays in which target cell populations are enriched prior to testing, the addition of the substrate (R123) directly to the blood sample combined with the segregation of the target cells by specific immunofluorescence provides the investigator an indication of *in situ* activity of circulating drug. Thus, CD56⁺ PBLs may prove useful as a surrogate target for monitoring multidrug resistance inhibitor activity *in situ*.

Introduction

The glycoprotein Pgp³ has been associated with the MDR phenotype displayed by tumor cells which are resistant to high levels of cytotoxic chemotherapeutic agents (1). It has been shown that Pgp can act as an ATP-dependent pump which moves candidate molecules from the cytoplasm to the exterior of the cell, and actively prevents the transit of such molecules through the plasma membrane into the cell (1). There are increasing amounts of data which suggest that the treatment of various cancers such as multiple myeloma or B-cell chronic lymphocytic leukemia with cytotoxic agents may select for variants which overexpress Pgp and thereby evade the effects of such drugs (2-5). As an aid in the development of MDR inhibitors, it would be desirable to have a surrogate clinical measurement of the degree of functional inhibition of Pgp, thus providing valuable information for the assessment of drug activity *in vivo*. The lipophilic dye R123 has been demonstrated to be a good substrate for Pgp, and there are numerous reports of the use of R123 for flow cytometric analysis of Pgp function in clinically derived tumor cell populations and selected cell lines (4, 6-10). Recent reports have identified certain normal human leukocyte subsets including CD4⁺, CD8⁺, CD34⁺, and CD56⁺ cells which express active Pgp as defined by cytometric assays of function in addition to immunophenotyping and PCR assays (10-15). Recently published data from kinetics experiments suggest that the CD56⁺ subset is the most uniform and most active with respect to the R123 efflux rate (13). These cells provide a potential source of *in situ* target for assessing MDR inhibitors *in vivo*, but as of this writing such efforts have been applied only to purified or enriched cell populations. We present here a method for detecting the effect of a novel and specific inhibitor of Pgp function, GG918 (16), on R123 loading of the CD56⁺ PBL subset in whole blood, and the application of this method to samples from p.o. dosed volunteers in a Phase I rising dose study. It is hoped that the development of an *in vivo* end point for drug effect will be useful in the pharmacological characterization of this and other inhibitors of MDR.

Materials and Methods

Reagents. R123 was purchased from Molecular Probes (Eugene, OR). RPMI (RPMI 1640 medium containing 25 mM HEPES) and PBS were purchased from GIBCO-BRL (Grand Island, NY). Sodium azide, propylene glycol, PI, BSA fraction V powder, and Histopaque 1077 were obtained from Sigma Chemical Co. (St. Louis, MO). Formaldehyde-fixed chicken erythrocytes (CRBCs) were purchased from Riese Enterprises

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³ The abbreviations used are: Pgp, P-glycoprotein; R123, rhodamine 123; PBL, peripheral blood lymphocyte; MDR, multidrug resistance; PI, propidium iodide; PE, phycoerythrin; HPLC, high pressure liquid chromatography; BP, band-pass filter.

(San Jose, CA). Murine IgG1-PE isotypic control was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL) and anti-CD56-PE-conjugated monoclonal antibody was obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). R123 was prepared from a stock solution in methanol (1 mg/ml) maintained at -20°C . The stock was diluted in RPMI before addition to the samples. The MDR inhibitor GG918 was dissolved in propylene glycol at a concentration of $800\ \mu\text{M}$, and dilutions were made in RPMI.

Blood Samples. Peripheral blood from healthy drug-free volunteers was collected by venipuncture in heparin-treated Vacutainer tubes (Becton Dickinson) and maintained at room temperature on a rocking platform prior to processing.

R123 Loading of PBLs. For loading of cells in whole blood, samples of heparinized whole blood were distributed to polypropylene tubes and appropriate concentrations of inhibitor or vehicle alone were added as 0.1 volume. The samples were then incubated at 37°C on a rocking platform for various times. Following pretreatment, R123 was added as 0.1 diluted volume to a concentration of 150 ng/ml unless otherwise specified, and incubation was continued for various times. The uptake of dye was interrupted at designated times by the addition of an equal volume of ice-cold PBS containing BSA at 0.1% (w/v) and sodium azide at 0.04% (w/v) (PBS/BSA) followed immediately by incubation in an ice bath for a minimum of 5 min. For loading of purified PBLs, the above steps were performed following the purification procedure detailed below.

Purification of PBLs. Drug- and R123-treated samples were layered on an equal volume cushion of Histopaque 1077 at 4°C and subjected to centrifugation at $1500 \times g$ for 30 min at 4°C . Following centrifugation, the PBL band was carefully collected with a transfer pipette and washed twice by centrifugation in 2 ml PBS/BSA at 4°C .

Staining of the CD56⁺ PBL Subset. Immediately before analysis, PBL samples were incubated with anti-CD56-PE monoclonal antibody in a 50- μl volume at a concentration of 20 $\mu\text{g/ml}$ for 30 min in an ice bath. Following the staining step, the samples were washed three times by centrifugation in 1 ml ice-cold PBS/BSA and suspended in 0.5 ml PBS/BSA containing PI at a concentration of 1 $\mu\text{g/ml}$.

Flow Cytometric Analysis. Samples were maintained in an ice bath until time of analysis. The data collection and analyses for the *ex vivo* modeling were performed on a Becton Dickinson FACStar^{Plus} flow cytometer equipped with an argon ion laser in the primary light path (60 mW at 488 nm; Ion Laser Technology, Salt Lake City, UT). Events were collected on a forward scatter trigger, and all fluorescence signals were processed by log amplifiers. Forward and side scatter data were not log amplified. R123 fluorescence was collected via a 530/30 BP following transit of a 560 short pass dichroic. PE fluorescence was reflected at the 560 short pass and collected using a 575/26 BP. The PI fluorescence signal was collected by transit of a 660/20 BP receiving signal from a 610 short pass dichroic. For each sample, 40,000 events were collected from a "live gate" of PI-negative cells, and list mode data analysis was performed using LYSIS II software (Becton Dickinson). Analysis gating was performed by using Cartesian plots displaying forward *versus* orthogonal light scatter to delineate the PBL population. Data from the PBL subset were then used to plot R123 fluores-

cence *versus* CD56-PE fluorescence and partition the CD56⁺ PBL subset. Histogram analysis of this subset was used to generate mean fluorescence values. Data collection and analyses of samples from dosed volunteers were performed on a Becton Dickinson FACScan flow cytometer using the same gating parameters and software. Both instruments were routinely checked for alignment, and target fluorescence values were set and checked using chicken CRBCs immediately before and after sample batches were run.

Clinical Study. This study was approved by the medical ethics committee of the Stichting Beoordeling Ethiek Bio-Medisch Onderzoek (Assen, the Netherlands) in accordance with the Declaration of Helsinki. All volunteers gave informed consent. Study entry included men, age range of 19–50, with no clinically significant abnormalities at screening; medical history, physical examination, clinical chemistry and hematology, urinalysis, indirect ophthalmoscopy, visual acuity, electrocardiogram, viral serologies, and urine drug screen. The study design was a rising single-dose, randomized, double-blind, placebo-controlled safety, tolerability, pharmacokinetic, and pharmacodynamic Phase I study.⁴ Parallel panels of four subjects (three GG918 and one placebo) received a single p.o. dose of the study drug at a given dose level on only one occasion. Immediately before and at 4 and 24 h after dosing, blood and serum samples were obtained for the CD56⁺ R123 fluorescence profile and GG918 measurement. The whole blood collected for R123 loading in CD56⁺ PBLs was divided equally and processed in parallel as duplicate samples.

HPLC Assay of Serum Drug Levels. The internal standard for the HPLC assay (a structural analogue of GG918) was solubilized in a 50:50 (v/v) mixture of acetonitrile and 50 mM ammonium acetate (pH 4.0). Serum samples (0.2 ml) were mixed with internal standard solution (0.05 ml) and 1.3% ammonium hydroxide solution (0.6 ml), and the aqueous mixture was extracted with 3 ml *tert*-butyl methyl ether. After thorough vortexing, the ether was retrieved and evaporated to dryness under nitrogen gas, and samples were reconstituted with the HPLC mobile phase prior to analysis. Calibration standards and quality control samples containing blank human serum with added amounts of GG918 were extracted and analyzed in a manner identical to the serum samples from dosed subjects. Chromatographic separation was performed at 37°C on a Hypersil ODS column (5 μm ; $250 \times 4.6\ \text{mm}$) from Chrompack (Middelburg, the Netherlands) using an acetonitrile:buffer mobile phase (45:55, v/v), with the buffer consisting of 50 mM ammonium acetate (pH 4.0) plus triethylamine (0.5 ml triethylamine added to each liter of buffer). The mobile phase was delivered at a flow rate of 1 ml/min using a Model 590 solvent delivery system from Waters Associates (Milford, MA). Fluorescence detection was performed with a Model 470 detector from Waters Associates using an excitation wavelength of 285 nm and emission wavelength of 510 nm. A solution of 1 N NaOH was added to the HPLC eluant as a postcolumn reagent to improve the fluorescence signal. The postcolumn reagent was delivered at a flow rate of 0.3 ml/min using a Model 610 Fluid

⁴ P. S. Wissel and B. M. Kerr, manuscript in preparation.

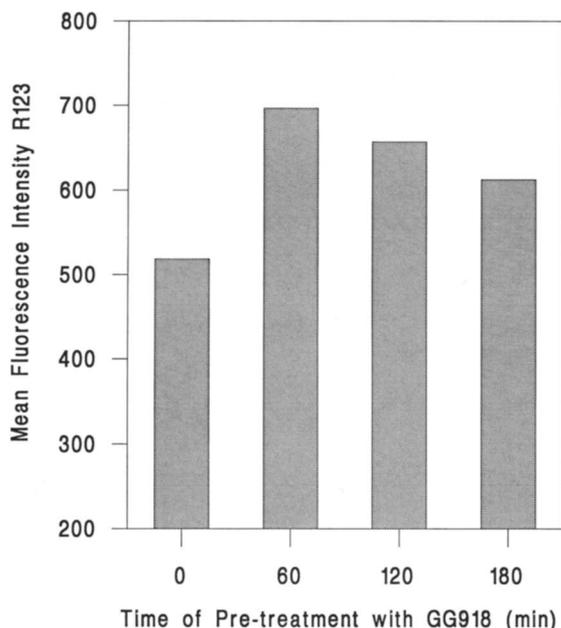


Fig. 1 Effect of time of drug treatment on R123 uptake in CD56⁺ PBLs. GG918 was added to heparin-treated whole blood samples to a concentration of 800 nM and incubated at 37°C for 0, 1, 2, or 3 h prior to the addition of R123 to a final concentration of 150 ng/ml. Incubation with R123 was continued for 15 min before termination by dilution in ice-cold PBS and the gradient-purification of PBLs for antibody staining and cytometric analysis.

Unit with a Model 600 Controller from Waters Associates. HPLC retention times were approximately 10 min for GG918 and 12.5 min for the internal standard. The assay range was 4.4–1780 nM.

Results

Ex Vivo Drug Treatment of Whole Blood Samples. To produce samples that would resemble those obtained from drug-treated individuals, we incubated aliquots of normal anticoagulated blood with fixed concentrations of GG918 for various times at 37°C. The effect of the drug on Pgp function was followed by flow cytometric analysis of R123 uptake in CD56⁺ lymphocytes. The treatment of the samples for 1 h was determined to produce a maximum effect on R123 loading in our standard protocol (Fig. 1). All subsequent incubations with the MDR inhibitor were carried out for 1 h at 37°C.

R123 Loading of CD56⁺ PBLs in Whole Blood. Many if not all of the published protocols for R123-mediated analysis of Pgp function were developed using purified cell systems (7, 8, 10). We felt it was necessary to characterize the kinetics of R123 loading of the target population in whole blood to ensure the optimization of detection of drug effects. Such experiments were carried out as time course analyses of R123 uptake in normal and drug-treated whole blood samples (Fig. 2). The uptake of R123 in whole blood by normal CD56⁺ PBLs approached maximum at 40 min while drug-treated cells continued to take up the dye over the entire assay period. By extending the dye loading time, the resolution of drug-treated *versus* normal

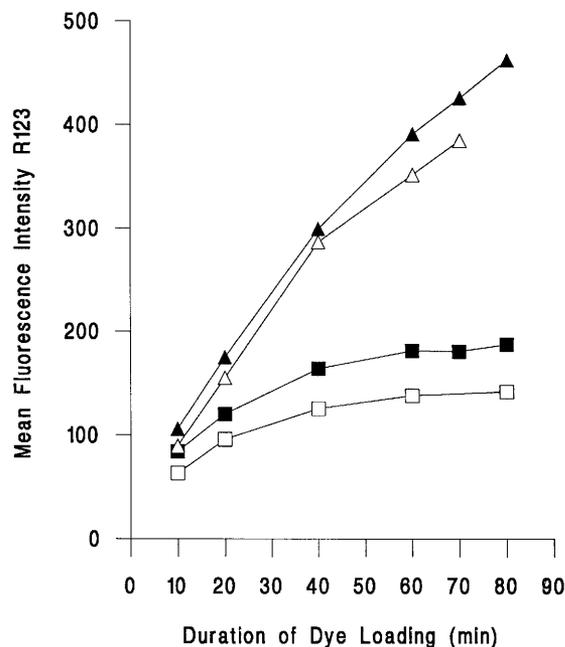


Fig. 2 Duration of the R123 uptake step and resolution of Pgp inhibition in CD56⁺ PBLs. GG918 was added to heparin-treated whole blood samples to a concentration of 100 nM and incubated at 37°C for 1 h prior to the addition of R123 to a final concentration of 150 ng/ml. Incubation was continued for various times before termination by dilution in ice-cold PBS and the gradient-purification of PBLs for antibody staining and cytometric analysis. Results from two different experiments are shown. □, ■, vehicle controls; △, ▲ 100 nM GG918.

PBLs was increased. Since the goal of this study was to develop a useful assay for patient samples, the R123 uptake step was limited to 60 min, and all subsequent experiments were carried out using this time period.

Previous reports have demonstrated a proportional relationship between the concentration of R123 and cellular fluorescence at equilibrium (17). Similarly, we found that the R123 fluorescence of normal as well as drug-treated samples was proportional to the R123 concentration (Fig. 3). Thus, the relative fluorescence of drug-treated and control samples was shown to be constant over the range of dye concentrations tested. When normal donor blood samples were treated with various concentrations of GG918, a dose-dependent increase in R123 fluorescence was demonstrated in the CD56⁺ PBL subset (Fig. 4). Under the conditions of the assay, a near maximum effect was observed in samples treated with 200 nM GG918.

Serum Level of GG918 and Fluorescence Analysis of CD56⁺ PBLs. The serum levels of total drug were determined at 4 and 24 h and demonstrated that interpatient variation of the C_{max} varied from 2- to 4-fold at a given dose (Table 1). The amount of drug present at 24 h (and predose) was routinely below the quantitative level of the assay, and fluorescence differences in the CD56⁺ cells were also negligible relative to the previous day's zero time point (data not shown). Changes in R123 fluorescence, expressed as relative to the zero time point, indicated that significant threshold levels of drug were obtained with the 20-mg dose. Representative analysis plots of list mode

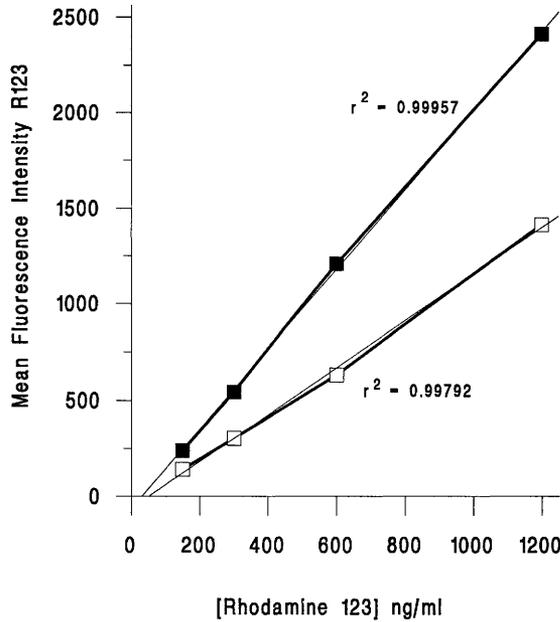


Fig. 3 Effect of R123 concentration on the detection of Pgp inhibition in CD56⁺ PBLs. GG918 was added to heparin-treated whole blood samples to a concentration of 100 nM and incubated at 37°C for 1 h prior to the addition of R123 to final concentrations of 150, 300, 600, or 1200 ng/ml. Incubation was continued for 1 h before termination by dilution in ice-cold PBS and the gradient-purification of PBLs for antibody staining and cytometric analysis. ■, 100 nM GG918; □, vehicle control.

data from patients F and A are presented in Fig. 5. Although the variability of percentage of change in mean fluorescence intensity relative to a given drug dose ranged from 2- to 4-fold, significant increases in R123 fluorescence were observed in all samples with serum concentrations above 50 nM.

Discussion

The effect of Pgp activity on the uptake as well as the efflux of such substrates as R123 and doxorubicin is well documented (7, 18–23). Coupled with flow cytometry, this technique has proven valuable in the assessment of responses of normal and malignant cells from bone marrow and peripheral blood to various drugs and reversal agents. Such experiments have generally been carried out on enriched cell populations (5, 22–25). For our purposes, it was important to rely on the existing concentrations and metabolic forms of the reversal agent to provide an accurate assessment of its effects *in vivo*. In the interest of developing a simple system for measuring the effect of GG918 on the Pgp activity in the CD56⁺ subset, we decided to examine only the dye uptake parameter. In such a system the inhibitor and the R123 could be added sequentially with minimal sample dilution. Such a preparation would more accurately represent samples from dosed individuals and would be more appropriate for the analysis of the effects of plasma and cellularity on drug availability. Unlike experiments in which the agent of interest is dosed into the test cell population, this protocol will allow measurement of the effect of the circulating drug (and possibly metabolites) as the only source of inhibitor. Although it may be possible to obtain data on efflux in this

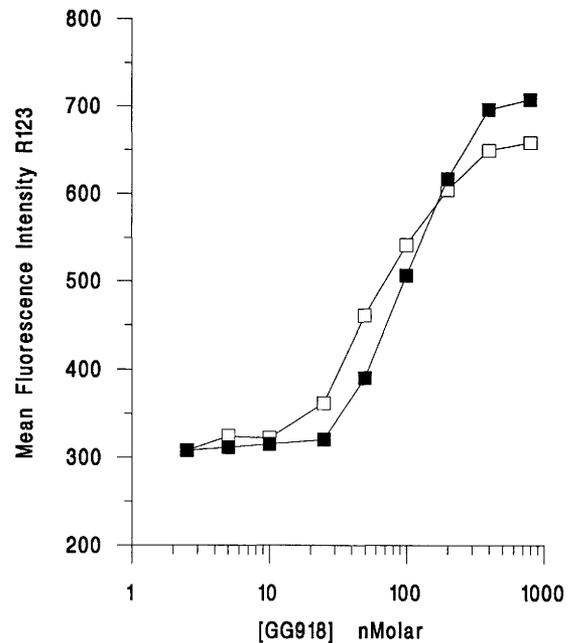


Fig. 4 Effect of GG918 dose on R123 uptake in CD56⁺ PBLs. The Pgp antagonist was added to heparin-treated whole blood samples at concentrations ranging from 2.5 to 800 nM and incubated at 37°C for 1 h prior to the addition of R123 to a final concentration of 150 ng/ml. Incubation was continued for 1 h before termination by dilution in ice-cold PBS and the gradient-purification of PBLs for antibody staining and cytometric analysis. The data shown were collected from two individuals in separate experiments. The R123 intensity values for volunteer 2 were normalized to those of volunteer 1 based on vehicle control values. ■, volunteer 1; □, volunteer 2.

Table 1 Patient serum C_{max} at 4 h and change in R123 fluorescence of CD56⁺ PBLs

Patient code	Dose administered GG918 (mg)	Serum concentration (nM)	Mean fluorescence intensity (arbitrary units) ^a	% Change in mean fluorescence intensity
A	0	0.0	256.1	2.5
B	0	0.0	243.7	-2.5
C	0	0.0	244.4	-2.3
D	0	0.0	264.9	6
E	0	0.0	242.3	-3.1
F	20	41.7	360.7	44.3
G	20	25.0	276.1	10.4
H	20	35.0	332	32.8
I	40	88.1	330.3	32.1
J	40	38.2	315.7	26.3
K	40	76.6	355.5	42.2
L	60	86.7	352.8	41.1
M	60	135.3	331.9	32.7
N	60	41.9	280.7	12.3
O	60	35.0	286.9	14.8
P	60	51.7	335.4	34.1
Q	60	138.2	344.4	37.8
R	80	98.4	321.1	28.4
S	80	61.1	335.1	34
T	80	82.2	312.2	24.9

^aValues represent means of duplicate samples processed in parallel.

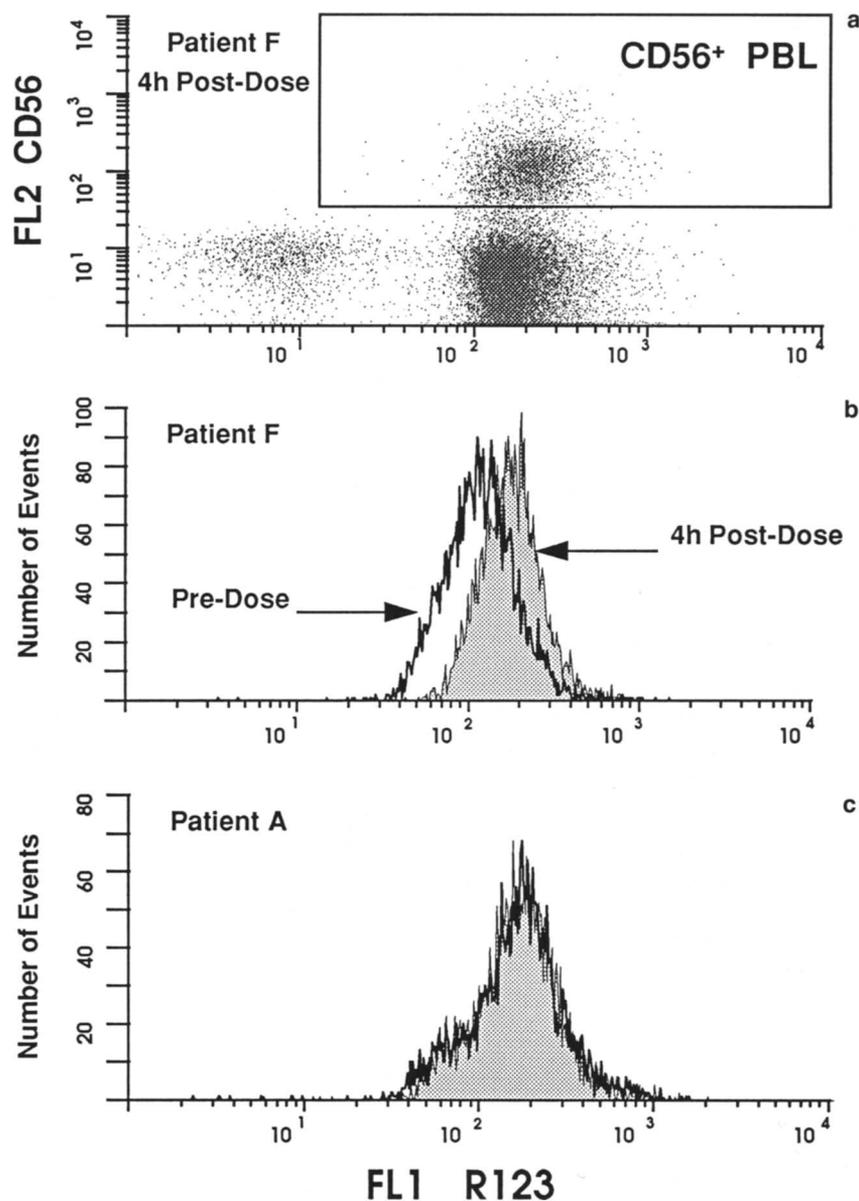


Fig. 5 Representative analysis plots of R123 fluorescence in CD56⁺ PBLs from dosed patients A and F. Data shown are PBLs previously delineated in plots of forward *versus* orthogonal light scatter. *a*, gating of CD56⁺ PBLs in a Cartesian plot of CD56-PE *versus* R123 fluorescence, patient F, 4 h postdose. *b*, superimposed R123 fluorescence histograms of CD56⁺ PBLs generated from data gated as in *panel a* for patient F. *Open trace*, predose; *filled trace*, 4 h postdose. *c*, superimposed R123 fluorescence histograms of CD56⁺ PBLs generated from data gated as in *panel a* for patient A (placebo). *Open trace*, predose; *filled trace*, 4 h postdose.

system, the washout of inhibitor in the course of sample preparation could have a profound effect on many such agents including verapamil. In contrast, this uptake assay could be performed on any agent regardless of its affinity for Pgp or other targets. Overall, this assay appears to provide a reasonable biological end point for evaluating the effect of MDR inhibitors in PBLs.

The variability in responsiveness relative to total drug concentration, however, is still elusive and will be addressed as the number of study participants expands. Understanding of the complete concentration *versus* response relationship will rely on additional measurements of free serum drug concentrations, activity of metabolites, and individual patient titration profiles of response to the drug. Additional studies addressing potential

differences in Pgp density on CD56⁺ lymphocytes relative to biological activity of MDR inhibitors might also provide further insight. Finally, the relative range of drug levels studied to date is limited; however, the shift in uptake in this assay is comparable to that seen by others in assays of enriched or isolated cell populations from peripheral blood (4, 22, 23, 25).

There has recently been a high level of clinical research activity involving pharmacological blockade of Pgp-mediated drug resistance (5, 7, 9, 16, 21, 22, 24–26). This area of research will likely expand over time as alternative mechanisms of drug resistance are discovered and pharmacological and immunological approaches to blockade are evaluated (27, 28). A guiding principle of drug development for this class of compounds will be the assessment of the drug effect. Clearly, the important

measurement of the drug effect will be the clinically measurable benefit derived by specific groups of cancer patients who are coadministered natural product-based chemotherapy. In the design of these "proof of concept" clinical trials, getting the dose of the modulator "right" will be a critical contribution by the preceding clinical pharmacology studies. If meaningful surrogate end points of activity can be validated, dose selection of the modulator in the patient will be better directed. To that end, further development of this assay may provide a significant tool for studies of MDR inhibitors in patients undergoing chemotherapy.

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