Quality Control of Multidrug Resistance Assays in Adult Acute Leukemia: Correlation Between Assays for P-Glycoprotein Expression and Activity

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We have compared multiple assays for the P-glycoprotein (Pgp/*MDR*1) phenotype in fresh and thawed adult acute leukemia to validate and quantitate measures for the expression and function of Pgp. The results are related to the Pgp-expressing KB8 and KB8-5 cell lines. The most sensitive assay was the measurement of modulation of the rhodamine 123 (R123) fluorescence by 2 μmol/L PSC833, followed by the modulation of the probe calcein-AM. We also found a good intralaboratory and interlaboratory correlation between the values of the R123/PSC833 assay for fresh as well as thawed samples. In addition, the effects of PSC833 on ^{3}H-daunorubicin (DNR) accumulation, DNR fluorescence, and ^{3}H-vincristine accumulation were very similar. The correlation between the DNR/PSC833 and R123/PSC833 test was $r = .86$ (N = 51). The modulation of drug accumulation by 8 μmol/L verapamil was the same as the PSC833 effect for DNR (117%, N = 21), but was higher for vincristine in every single case (161% v 121%, N = 22; $P < .001$), indicating additional verapamil effects, not related to Pgp. The correlation of the staining of viable cells for Pgp with the monoclonal antibody MRK16 was $r = .77$ (N = 52) for the R123/PSC833 functional test and $r = .84$ (N = 50) for the DNR/PSC833 test. From these results it could be calculated that a maximal increase of the mean DNR accumulation of about 50% can be achieved by blocking Pgp pump activity with PSC833 in leukemic blast samples with the highest mean Pgp expression. Subpopulations of blast cells with higher Pgp activity are likely to be present. Their relevance has to be studied further.The methods outlined here allow the reliable, quantitative monitoring of the Pgp/*MDR*1 phenotype in leukemias in multicentered, clinical Pgp modulation studies.

ACUTE MYELOID leukemia (AML) in adults is responsive to chemotherapy in approximately 65% of the patients. To date only 30% to 35% of the patients can be cured with chemotherapy alone because of the emergence of malignant cell populations, which are resistant to all clinically available anticancer drugs. One cause of tumor cell resistance to natural product related anticancer agents, such as the anthracyclines, vinca alkaloids, and etoposide, may be the overexpression of the *MDR*1-encoded plasma membrane drug transporter P-glycoprotein (Pgp/*MDR*1).[1,2]

If one focuses on adult acute leukemic disorders such as AML and acute lymphocytic leukemia (ALL), there is not yet a curative standard treatment.[3] One factor studied extensively as a possible contributor to chemotherapy failure in leukemias is the Pgp/*MDR*1 phenotype. Such studies have provided quite firm evidence that Pgp/*MDR*1 overexpression is relatively abundant in primary, treatment refractory and in relapsed leukemias.[4,5] Moreover, complete remission rates were significantly lower in de novo AML overexpressing *MDR*1 mRNA or Pgp.[4-7] However, a recent overview of published data for AML concluded that the effect of Pgp on the outcome was weak (relative risk of 0.68 for complete remission).[8] This analysis included the results of the two largest studies performed, published as abstracts, which report that Pgp expression is not a prognostic factor in AML.

Many of these studies rely on mRNA detection (Northern blotting, RNase protection, or polymerase chain reaction [PCR]) and/or detection of Pgp protein (immunocytochemistry, flow cytometry, immunoblotting) and express the results as positive or negative by choosing an arbitrarily set cut-off point.[9] However, drug-resistance levels are more likely to be a continuous variable rather than that tumor cells are simply positive or negative for a particular resistance mechanism. A second point to be considered is that the establishment of the relevance of the function of Pgp in clinical multidrug resistance (MDR) will probably require multicentered prospective clinical studies. Results of MDR assays have to be correlated with patients' responses to treatment. Therefore, "to be successful, a concerted effort will be needed to standardize reporting and quality control between participating laboratories".[10] The need for such quality control studies in the MDR field has become increasingly clear from two interlaboratory MDR methods workshops held in 1994, one in France and one in Memphis, TN. Major discrepancies were found in the results of Pgp/*MDR*1 detection in the same leukemic samples between different laboratories, when each laboratory used its own MDR detection method according to local protocols, including widely used techniques such as immunocytochemistry and reverse transcriptase-PCR (RT-PCR).

The purpose of this work was threefold: first, to establish the feasibility and reproducibility of functional Pgp assays in adult acute leukemias in an interlaboratory setting; second, to study the reproducibility of functional Pgp assays in fresh and thawed leukemic samples. Third, we wished to establish a quantitative correlation between the expression level of Pgp and its drug transport function in leukemic blasts. Such data may give us insight into the maximal effect of the expression of Pgp on the intracellular drug concentrations in leukemic blasts. Moreover, quantification of the effect of a Pgp modulator on the intracellular drug accumulation in

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leukemic cells from individual patients may provide a rationale for clinical application of such compounds.

We have studied the correlation between the functional rhodamine (R123) and daunorubicin (DNR) accumulation tests performed in two laboratories in fresh as well as thawed leukemic samples. In addition, the correlation between fluorescence-based flow cytometric functional assays (Rh123, calcein-AM, and DNR) with the radioactive drug uptake (DNR and vincristine [VCR]) as well as the correlation of these functional assays with the Pgp expression, measured by an optimalized flow cytometric assay with the monoclonal antibody (MoAb) MRK16, was studied.

MATERIALS AND METHODS

Patients. Peripheral blood (PB) or bone marrow (BM) was collected in heparinized glass tubes before treatment after patients had given informed consent. Fifty-nine samples from 57 patients (55 AML and 2 ALL, 2 AML at presentation and after relapse) were analyzed. Mononuclear cells were isolated on Lymphoprep (Ficoll-Paque; Pharmacia LKB, Uppsala, Sweden) by centrifugation for 25 minutes at 2,000 rpm. Interphase cells were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM), buffered with 20 mmol/L HEPES, pH 7.4 (without phenol red) and supplemented with 10% fetal calf serum (FCS). Samples contained at least 75% blasts, except patients 3 (34%), 14 (67%), 19 (68%), 28 (68%), 29 (71%), 35 (67%), 37A (66%), 39 (50%), 40 (63%), and 53 (60%). Samples were analyzed for Pgp function on the same day (within 4 to 6 hours) or frozen in medium with 10% FCS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen. For analysis the frozen samples were rapidly thawed in a waterbath (37°C) and immediately diluted in 10 mL of medium with 40% FCS, prewarmed to 37°C. The cells were centrifuged and resuspended in medium with 10% FCS for further experiments. When thawed cells were used for functional flow cytometric assays they were preincubated for 60 minutes in medium with 40% FCS at 37°C to allow recovery of metabolic activity, before resuspension in medium with 10% FCS. These cells had usually a trypan blue viability greater than 90%.

Radioactive drug accumulation. Drug accumulations were performed as described in detail before.[11] In short, 1 to 1.5 $\times$ 10^6 leukemic cells were incubated in medium with 0.005% DNaseI (Boehringer, Mannheim, Germany) and 0.5 μmol/L ^{3}H-labeled DNR or VCR (Amersham, Little Chalfont, UK) for 75 minutes at 37°C. Pgp-mediated drug transport was blocked by coincubation with 2 μmol/L SDZ PSC833 (gift of Sandoz, Basel, Switzerland) or 8 μmol/L verapamil (Sigma Chemical Co, St Louis, MO). Direct drug binding to cells at 0°C was subtracted from drug accumulation values.[12] This direct binding was less than 2% of experimental values.

Flow cytometric drug accumulation. Cells (0.3 to 0.7 $\times$ 10^6) were incubated for 75 minutes at 37°C in 2 μmol/L DNR (Sigma) or 200 ng/mL R123 (Sigma) or for 10 minutes at 37°C with 0.5 μmol/L calcein-acetoxymethylester (calcein-AM; Molecular Probes, Eugene, OR) with or without 2 μmol/L PSC833 (Sandoz) or 200 μmol/L genistein (Sigma; with control DMSO). All the data are ratios of drug fluorescence with modulator divided by drug fluorescence without modulator after subtraction of the fluorescence of the blanks (incubated with drug on melting ice and washed immediately; this blank was less than 2% of experimental values). Fluorescence was analyzed with FACScan flow cytometers (Becton Dickinson Medical Systems, Sharon, MA, USA)[13] at the two different locations. Dead cells were gated out by scatter characteristics.[14]

Flow cytometric P-glycoprotein detection. MRK-16 antibody (Dr T. Tsuruo, Tokyo, Japan) was chosen because it recognizes an outer epitope of Pgp, allowing the staining of viable cells. Previously we have compared MRK16 with 4E3 antibody in 20 AML samples and did not find different results.[15] Neither did we see an increased reaction of MRK16 after neuraminidase treatment in 6 of 7 AML. In one AML and in KB3-1 Pgp–negative cells, we found an aspecific increase in MRK16 binding after neuraminidase treatment, which therefore was not applied in this group of leukemias. Viable Pgp expressing KB8-5 cells were titrated with MRK-16 to study binding characteristics. It appeared that saturation of binding was not yet reached at 100 μg/mL MRK-16, even when the cells were incubated for 60 minutes at room temperature (see Fig 1). We think that the increase in binding with increasing antibody amounts was specific, since it was not seen in KB3-1 cells. Because different batches of isotype control antibody differed in binding to KB3-1 and AMLs we selected a batch of isotype control with low binding. Two AMLs (nos. 29 and 40) still were considered not evaluable because of a rather high binding of isotype controls. Frozen leukemic samples were rapidly thawed and washed and resuspended in phosphate-buffered saline (PBS) containing 1% rabbit serum and 0.1% goat serum (Dakopatts, Copenhagen, Denmark). Antibodies were diluted in the same medium. Based on Fig 1 we used 25 μg/mL MRK-16 or nonrelevant mouse IgG2a (Sigma) at room temperature (60 minutes) to label the leukemic samples. After washing, the cells were incubated for 45 minutes in the dark with rabbit-antimouse fluorescein-isothiocyanate (1:100) (Dakopatts). Fluorescence was measured on a FACScan. Dead cells were gated out by scatter characteristics. The mean fluorescence of MRK-16-labeled cells divided by IgG2a-labeled cells was calculated. As a control KB3-1, KB8, and KB8-5 cells were analyzed, which had a mean fluorescence ratio (MRK16: IgG2a) of 1.05 $\pm$ 0.18, 2.9 $\pm$ 0.4 and 12.4 $\pm$ 3.8, respectively (mean $\pm$ SD of three experiments on different days).

RESULTS

Fresh leukemic samples were obtained from both Hematology Departments and stored on ice in 10% FCS containing medium. From the functional assays described in the literature we chose flow cytometric determination of R123, calcein-AM, and DNR fluorescence and compared it with an accumulation assay of ^{3}H-DNR and ^{3}H-VCR with or without 2 μmol/L PSC833. These choices are based on previous experience comparing different probes in Pgp and multidrug resistance protein (MRP1) overexpressing cell lines,[13] showing that R123 and calcein-AM are sensitive Pgp-probes,

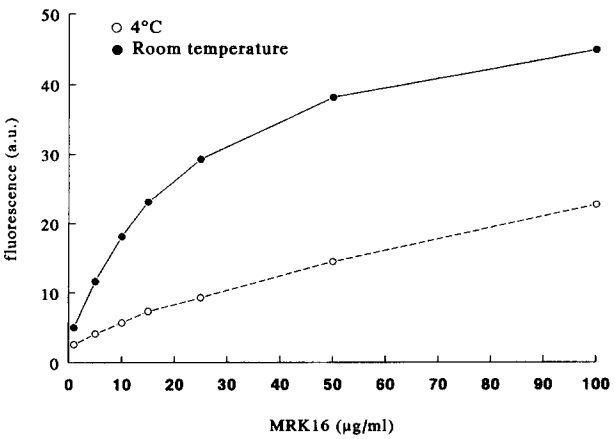

Fig 1. Labeling of KB8-5 cells with MRK-16 on ice and at room temperature.

whereas DNR is a more clinically relevant probe. For R123 we chose a short-term (75 minutes, 37°C) accumulation assay, instead of studying R123 efflux, because of its specificity for Pgp and not MRP1-mediated transport. This assay is still sensitive enough to identify KB8 cells (≈twofold resistant) as Pgp MDR cells.[13] Moreover, a short assay time may limit a potential decrease of metabolic activity and loss of viability of leukemic blasts during the assay.[13,16] Nevertheless, it may very well be that an efflux assay in AML is in practice a useful alternative. Most important is probably the right choice for the combination of probe (dye) and modulator (see also Discussion).

Reproducibility of the functional assays. The effect of freezing in liquid nitrogen (N2) and thawing on the outcome of the functional flow cytometric assays was determined in leukemic samples. The results (Fig 2A and B) show a good correlation, especially for the R123/PSC833 test. An interlaboratory comparison in leukemic samples showed essentially the same trend (Fig 3A and B). The correlation of the results between both institutes again was high for the R123/PSC833 test (r = .87, N = 12). For the DNR/PSC test the correlation was less (r = .55, N = 13). The better correlation for an R123 than for a DNR functional test is probably at least partly related to larger modulatory effects on the R123 than on the DNR accumulation. This makes the test more sensitive and possibly less subject to variations in experimental conditions.

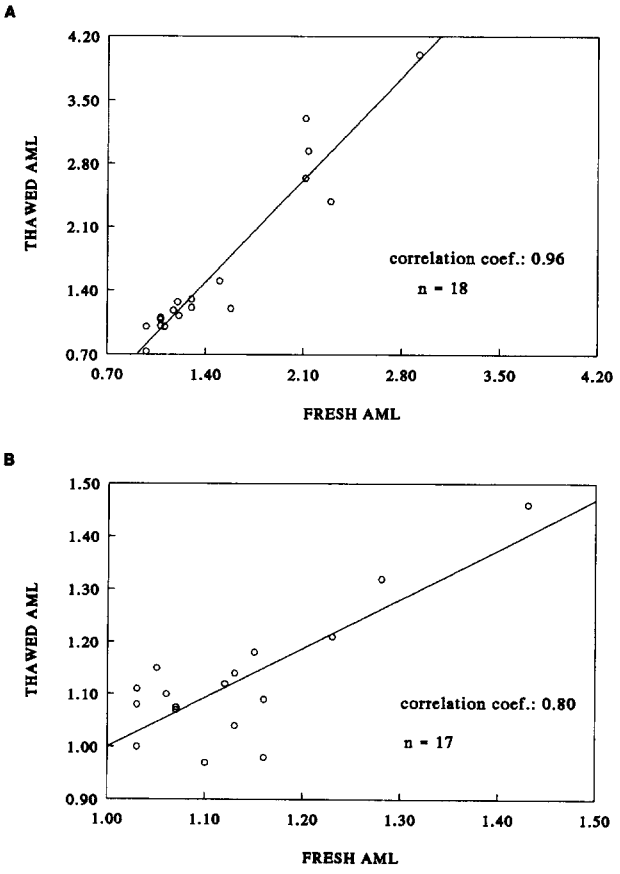

Fig 2. Data are ratios of mean R123 (A) or DNR (B) fluorescence with PSC833 (2 μmol/L) divided by mean R123 or DNR fluorescence without PSC833 in fresh and thawed leukemias.

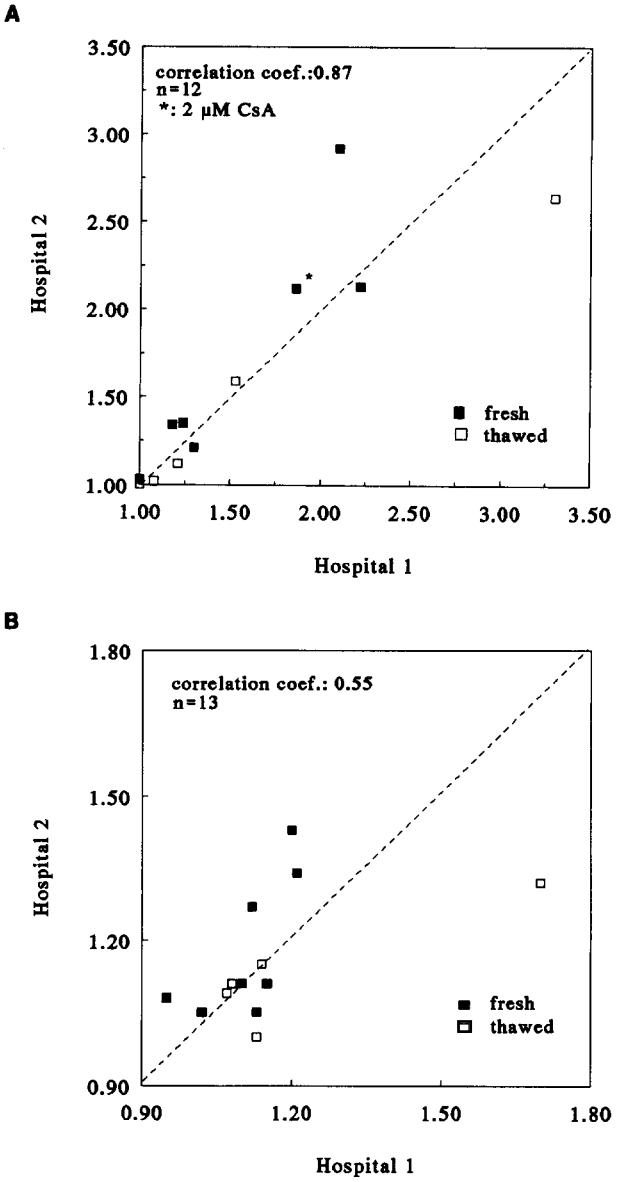

Fig 3. Data are ratios of mean R123 (A) or DNR (B) fluorescence with PSC833 (2 μmol/L) divided by mean R123 or DNR fluorescence in leukemias. Comparison between two institutes. *2 μmol/L CsA: in this sample 2 μmol/L cyclosporin A was used in hospital 1.

Results of the functional and Pgp assays. The Pgp modulation and Pgp expression (MRK16) values of a representative selection of leukemic samples are shown in Table 1. In Table 1 all the flow cytometry data (columns 4 through 6) show the mean fluorescence values of the cell population. Subpopulations are indicated only when they were clearly visible. However, these data (percentages and fluorescence values) are estimations because in almost all the cases subpopulations had extensive overlaps. Subpopulations were observed with three different assays. First, the clearest indica-

Table 1. Modulation of Drug Fluorescence/Accumulation by PSC833 and Verapamil and Pgp, Measured With MRK16 in Representative Leukemic Samples

Patient No.	^{3}HDNR/PSC (verapamil)	^{3}HVCR/PSC (verapamil)	DNR/PSC	R123/PSC	Pgp/MRK16
7	1.05*	0.99	1.04	1.02	1.3
8	1.34	1.35	1.34	2.35	5.0
9	1.08 (1.03)	1.14	1.05	1.05	1.7
10	1.32 (1.34)	1.20 (1.83)	1.27	2.12	3.9
11	1.36 (1.37)	1.32 (1.64)	1.12	2.03	8.7
12	ND	ND	1.43	2.92	9.5
13	ND	ND	1.11	1.34	2.5
14	1.17 (1.20)	1.17 (1.53)	1.08	1.38 50% = 3.02	2.9
15	ND	ND	1.03	1.32 20% = 5.32	2.6 (few % higher)
27	1.09 (1.10)	1.01 (1.38)	1.05	1.20	3.0
28	1.00 (1.02)	1.10 (1.63)	1.06	0.95	1.8
29	1.22 (1.17)	1.11 (1.43)	1.14 subp	1.19 subp†	not ev‡
37A	ND	ND	1.05 (N2)	1.7 50% = 4.4 (N2)	4.0
37B	1.45 (1.41)	1.73 (2.08)	1.35	40% = 2.1 50% = 7.3 10% = 1	93% = 7.2 6% = 0.6
47A	ND	ND	1.15	1.40 20% subp†	3.2 20% = 4.0
57	ND	ND	ND	1.30 subp gen† (N2)	6.4

Abbreviation: ND, not done.

* Data are ratios with/without modulator.

† subp (gen), subpopulation (seen with genistein; see Discussion).

‡ MRK16 not evaluable (high IgG binding, cell clumps), also see text. Nos. 37A and 57 functional tests from a thawed sample.

tions came from the R123 test showing a subpopulation of cells on exposure to PSC833 in some leukemias. Secondly, previously we have found that the isoflavonoid genistein may decrease the retention of DNR and R123 in Pgp overexpressing cells, indicating increased efflux by an as yet unknown mechanism (unpublished results, March 1995), whereas it inhibited DNR efflux by MRP1.[17] A similarly highly decreased R123 retention by genistein was also found in a subpopulation of cells in a number of leukemic samples (indicated in Table 1 by "subp gen"). Thirdly, subpopulations can be seen in some of the higher Pgp expressing leukemias with MRK16. In the analyses (all the figures and other tables) always the mean values of the whole population of leukemic cells are plotted, because the data on subpopulations are too scanty to be analyzed.

Comparison of the functional assays. Tables 2 and 3 and Fig 4 show the comparisons between the different functional assays. In Table 2 the modulation of DNR and VCR accumulation by PSC833 or verapamil is given. Both modulators were equally active except for verapamil in combination with VCR which gives a higher modulation than 2 μmol/L PSC833 can achieve. Verapamil also modulated VCR accumulation in several leukemias in which no R123 modulation or Pgp could be detected. It is of interest that the radiolabeled and fluorescent DNR accumulation test provided the same results because both methods are used by several groups. However, it is clear that the mean DNR modulation in the majority of leukemias is small (0% to 50% increase by Pgp inhibition). Figure 4 shows the correlation between the R123/PSC833 and DNR/PSC833 assay (r = .86, n = 51). In this figure the corresponding values for the low resistant KB8 cells are shown for comparison, illustrating that values for leukemias scatter around this value.

Table 3 shows the results of some comparisons between R123 and calcein-AM as Pgp functional probe. In this limited number of samples calcein-AM does not seem to be as sensitive as R123.

Correlation between the functional and Pgp assays. Figure 5A and B show the relation between Pgp expression as measured with MRK16 and the modulatory effects of PSC833 on the R123 (r = .77, N = 52) and DNR (r = .84, N = 50) fluorescence. The correlation between MRK-16 staining and the modulation of radioactive drug accumulation (the latter all done on fresh samples) was as follows:

Table 2. Comparison of Modulator Effects on Drug Accumulation in Leukemic Cells

Drug/Modulator	Ratio [with:without modulator]
^{3}H-daunorubicin/PSC833	1.16 ± 0.13 (N = 34)
^{3}H-vincristine/PSC833	1.22 ± 0.19 (N = 34)
^{3}H-daunorubicin/PSC833	1.17 ± 0.14 (N = 21)
^{3}H-daunorubicin/verapamil	1.17 ± 0.15 (N = 21)
^{3}H-vincristine/PSC833	1.21 ± 0.19* (N = 22)
^{3}H-vincristine/verapamil	1.61 ± 0.24* (N = 22)
^{3}H-daunorubicin/PSC833	1.16 ± 0.13 (N = 34)
FACSdaunorubicin/PSC833	1.12 ± 0.11 (N = 34)

Data are ratios of drug accumulation with/without modulator. Data are from samples where direct comparisons have been made; therefore, the number of samples varies per comparison.

* Significantly different (t-test): $P < .001$.

Table 3. Comparison of R123 and Calcein-AM To Probe Pgp in Leukemias

Patient	R123/PSC833	Calcein-AM/PSC833
18	2.04 (N2)	0.78 (N2)
43	1.04	1.0
44	1.56 (N2)	1.4 (N2)
45	1.08	0.91
46	3.70	1.63
47	1.40	1.14
48	1.50	1.20
49	1.30	1.17
50	1.20	1.03
50	1.27 (N2)	0.93 (N2)
51	1.18	0.86
52	1.05	0.96
53	1.06	0.94
54	2.70	3.36

Data are ratios of dye accumulation with/without PSC833. R123 accumulation was 75 minutes and calcein-AM accumulation was 10 minutes at 37°C.

Abbreviation: N2, thawed leukemia.

for ^{3}H-DNR/PSC833 r = .83 (N = 32); ^{3}H-DNR/verapamil r = .92 (N = 21); ^{3}H-VCR/PSC833 r = .73 (N = 31); ^{3}H-VCR/verapamil r = .71 (N = 22).

DISCUSSION

The aim of this study was to contribute to standardization of Pgp/*MDR*1 detection methods in leukemias and to establish a quantitative relationship between Pgp expression and effects on the intracellular drug accumulation. The need for such a study was felt by us, because we wished to devise experimental protocols for Pgp/*MDR*1 detection to monitor clinical MDR modulation trials. A survey of the vast literature on this subject did not clearly show which methods and reagents should be used as exemplified by citing two recent reports: "Whichever method is chosen to quantitate *MDR*1

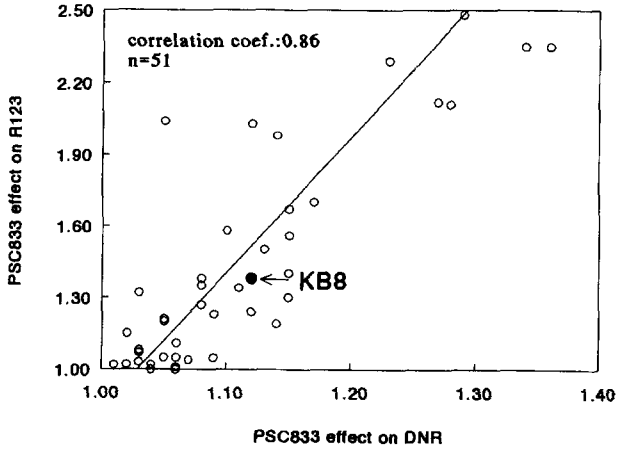

Fig 4. Relation between PSC833 (2 μmol/L) effect on mean DNR and R123 fluorescence in leukemias. The line is the best linear fit. For reference KB8 cells are shown.

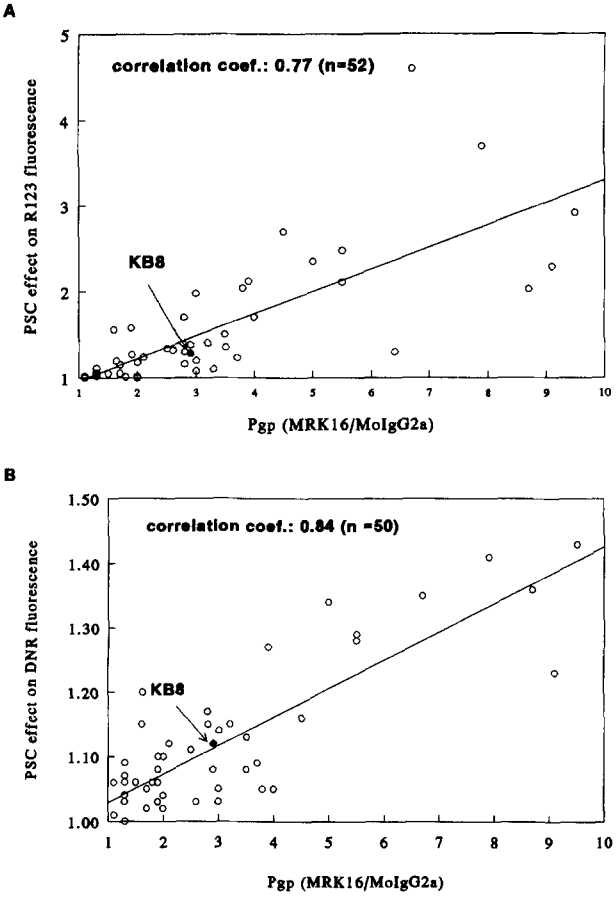

Fig 5. Relation between PSC833 (2 μmol/L) effect on mean R123 (A) or DNR (B) fluorescence and Pgp expression, as measured with MRK16 in leukemias. The line is the best linear fit. For reference KB8 cells are shown. (A) r = .77 (P < .005); (B) r = .84 (P < .001).

expression levels, a critical requirement is the use of consistent standards among different laboratories, both in terms of the drug-resistant cell lines used to calibrate the assays, the percent of positive cells considered meaningful, and the differences in *MDR*1 expression that are considered significant."[9] Further, "At the current time, no standardized method has been agreed on for detecting and quantifying *MDR*1 expression in tumor specimens. However, in addition to the issues of sensitivity and specificity, any detection method must also not be too difficult, time-consuming, or costly for routine work in diagnostic pathology departments if such analyses will ever be expected to exit from the province of the research laboratory".[18] Even reports published in 1995 on the role of Pgp in leukemias repeatedly state that the results suggest the need for a clear standardization of Pgp detection methods.[19-21] The need for standardization and quality control of MDR detection methods was clearly demonstrated by an MDR detection workshop held in Memphis, TN, in 1994, showing that the results of determination of Pgp/*MDR*1 expression in three leukemic samples widely differed among methods and laboratories.[22] Consensus recommendations which have been formulated during that workshop and are followed in our study are discussed below.

First, multiple detection methods using a variety of techniques are used. Second, for flow cytometric Pgp detection an antibody recognizing an outer epitope (MRK16) was used. Third, the batch MRK16 used was calibrated using well-characterized Pgp expressing cell lines with low Pgp expression (KB8 and KB8-5). Fourth, the results were not expressed as percentage positive cells because an overlap between the isotype control distribution and the MRK16 distribution was always found. Therefore, the ratio of both means was reported instead and if a clear indication for a subpopulation was seen, it was reported. No arbitrary cut-off levels for positivity were assigned as had been recommended by the workshop study.

In this report we extensively compare different functional Pgp assays. For such assays the condition of the cells is critical. In principle, the functional assays performed in this study, are done on freshly (Ficoll) isolated cell populations, kept in 10% FCS containing medium and when assayed in the other laboratory transported on ice. The assays were performed as soon as possible (within 6 hours after the sample had been taken from the patient), to minimize the possibility that dye efflux is compromised[16] (Schuurhuis et al, unpublished data). For the comparisons of fresh and thawed material, the leukemic samples were rapidly thawed and diluted in warm medium containing 40% FCS and allowed to recover for 1 hour at 37°C, according to the workshop recommendations. The functional assays were performed by comparing a drug accumulation with and without modulator (we chose PSC833 or verapamil), instead of measuring drug or dye accumulation as such. This was based on extensive cell line experiments using KB3-1, KB8, and KB8-5 cells[13] and on the workshop recommendations.[22] In accordance with the cell line experiments[13] the PSC833/R123 combination was the most sensitive functional Pgp assay, most likely because the ratio between passive and Pgp-mediated active R123 efflux is in favor of the latter, despite the fact that also R123 may have some unfavorable properties, such as a higher lipid solubility than daunorubicin. The theoretical requirements for an optimal MDR probe have been discussed by us elsewhere.[23] Probably because of the high sensitivity of the R123 test, we find a good correlation between the results of the two laboratories and between fresh and thawed samples. Moreover, because R123 accumulation appeared to be an insensitive way to measure the transport function of the MRP1 protein, it is selective for Pgp.[13] Based on these observations, R123 is the only probe of the presently tested dyes that we would recommend at this moment for use in a more routine setting in leukemic blasts.

If a modulator other than PSC833 is chosen (eg, cyclosporin A) the validation of such data (eg, in thawed leukemias) has to be shown first. Moreover, even a modulator such as cyclosporin A, which is structurally highly related to PSC833, may give worse correlations with Pgp expression because of additional effects (Sonneveld et al, unpublished results).[24] Calcein-AM in combination with PSC833 appears to be a probe with somewhat less sensitivity than R123, but may not be as Pgp specific. Our data are not yet sufficient to prove that thawed material is suitable for testing with this probe. This remains to be done if one wishes to use calcein-AM to probe the Pgp function in thawed leukemic samples.

The comparison of two different drug accumulation assays for DNR (flow cytometric and radioactive method) that are both used in many laboratories gave very similar values for the effects of the modulators PSC833 and verapamil. These results greatly enhanced our confidence that the values we found are true modulator effects and not caused by, for example, quenching of the fluorescence of the probe. The fact that the modulator effects on R123 or DNR fluorescence highly correlated with MRK16 binding to the leukemic samples is further evidence that both assays are specific for Pgp activity. One sample from a patient with a low blast count had a high Pgp expression with a rather low PSC833 modulation of R123 fluorescence (determined in a thawed sample) (no. 57). Since in this sample no other functional test was done the reason for the discrepancy is unknown.

Because we had to use a high concentration of MRK16 to obtain the required sensitivity, binding of isotype controls in some of the samples was also considerable and we found that it may vary between different suppliers. A further improvement might therefore be the use of $F(ab')_2$ fragments of MRK-16 to lower the aspecific binding. In addition, there is clearly a place for anti-Pgp antibodies with higher affinity to improve Pgp detection by flow cytometry and immunocytochemistry.[25]

Another conclusion from our data is that VCR seems to be only marginally more sensitive than DNR as a probe for Pgp function in leukemias. Moreover, in the combination VCR with 8 μmol/L verapamil, an additional effect that is not related to Pgp is detected. Whether this suggests the presence of another drug transporter in leukemias is an open question. In any case, it clearly indicates that for every new combination of probe (drug) and modulator one has to establish its specificity and sensitivity in detecting the action of drug pumps such as Pgp. In general, the determination of MDR parameters using different drugs and techniques as performed here and in previous studies[15,26] greatly enhances the possibility of identifying putative experimental errors or other non-Pgp related effects. If one parameter (eg, MRK16) is not in accordance with another (R123/PSC833), these tests can be repeated from frozen material to exclude an incidental experimental error.

An important aspect of Pgp tests is the issue of heterogeneity in its expression within a cell population. Whenever a clear subpopulation was seen this has been reported in Table 1. Such subpopulations were most often seen in the R123 assay, sometimes with PSC833 but more frequently with genistein as a modulator. Because this genistein effect has not been fully investigated and explained using model cell lines,[27,28] we do not want to overemphasize these data presently. However, the test may be promising for revealing certain subpopulations.

In some cases, subpopulations are seen with the MRK16 test, but only clearly when there exist large differences between the populations (eg, patients 15 and 37B). In almost all the samples it would need a further mathematical analysis to define the highly overlapping subpopulations. We have used Kolmogarov-Smirnov (KS) statistics for the analysis of some critical AML samples and found a good correlation with the mean shift as reported here, in agreement with a

recent report.[29] Thus, we think that for the present purpose the way of reporting our data is adequate. However, it may very well be that in studies into the relevance of small subpopulations of Pgp expressing cells for the clinical outcome of AML, the more objective KS method is needed. In such studies intended at focusing on subpopulations of Pgp-expressing cells, eg, the relevance of their presence in primary disease for treatment outcome or the increase after relapse, we would recommend including an R123 test. In addition, one may identify subpopulations of cells by combining Pgp functional assays with immunophenotyping of the antigenic make-up of the cells, eg, $CD34^{+}$[7,19,29,30] or $CD7^{+}$.[31]

Finally, this study allows us to calculate the maximal effect of Pgp expression on the mean DNR uptake in the leukemic blasts and to relate this to a certain value for MRK16 binding. The maximal effect of inhibition of the Pgp function by PSC833 was about a 50% increase in the mean DNR accumulation. Because 2 μmol/L PSC833 is able to completely reverse the DNR accumulation deficit in KB8 and KB8-5 cells that have a similar Pgp expression level as leukemic cells,[13] this is the maximal effect to be expected on the DNR accumulation brought about by complete Pgp inhibition in vivo, if one does not take into consideration the effect of in vivo modulation of the pharmacokinetics of the cytostatic agent.[32] Notably, based on cell-line data, such an increase of DNR accumulation might have a more than proportionate effect on cell survival, possibly because drug distribution phenomena may underestimate the effect of drug transporters at the target concentration.[33,34] Some data suggesting similar phenomena in leukemia have been reported.[15]

Another issue is that small subpopulations of leukemic blasts with higher Pgp expression (see before) may undergo larger effects of efficient Pgp inhibition on the DNR accumulation. The relevance of such subpopulations in clinical MDR modulation trials is still completely unknown.

To obtain information on the value of the present parameters in predicting clinical response in the leukemia patients it is necessary to perform a prospective study including a test for the Ara-C sensitivity, because all of these patients received Ara-C containing chemotherapy.[15,35] Moreover, Pgp tests have to be combined with measuring *MRP*1 mRNA, MRP1 protein with MoAbs now being available,[36] and MRP1 functional tests.[37] In practice, the presently recommended R123/PSC833 flow cytometric Pgp test is sufficiently sensitive and specific to perform on an interlaboratory basis and can be combined with MRK16 staining and with the above-mentioned assays for MRP1-mediated resistance.

In conclusion, this study defines critical factors for practical Pgp-mediated MDR testing in leukemias and provides a way of dealing more quantitatively with the effect that Pgp expression might have on cytostatic drug accumulation in this disease.

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