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# Clinical Application of a Rapid, Functional Assay for Multidrug Resistance Based on Accumulation of the Fluorescent Dye, Fluo-3

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**A rapid and simple functional assay for P-glycoprotein (Pgp) using flow cytometry to measure the accumulation of the flurophore fluo-3 has been applied to samples from patients with B-cell chronic lymphocytic leukaemia (B-CLL). Peripheral blood lymphocytes from 37 patients with B-CLL were studied for Pgp. Pgp expression, using MRK-16, a monoclonal antibody recognising an external surface epitope of Pgp, was detected in 92% of patients with B-CLL. The functional assays for Pgp expression were positive in 78 and 59% of patients using the fluo-3 and doxorubicin (dox) assays, respectively. When compared with the MRK-16 assay, the fluo-3 assay had a sensitivity of 82% compared to a sensitivity of 56% for the dox assay ($P = 0.004$). The specificity of the fluo-3 and dox assays could not be evaluated because of the low number of MRK-16 negative CLL cells.**
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## INTRODUCTION

CLASSICAL MULTIDRUG resistance (mdr) is the phenotype conferred on cancer cells by the expression of p-glycoprotein (Pgp). Pgp functions as an ATP-dependent efflux pump for a variety of structurally unrelated drugs [1].

We have previously shown that the drug-sensitive T-lymphoblastic leukaemia cell line CEM-CCRF accumulates the calcium fluorophore fluo-3, 20-fold faster than the multidrug-resistant variant CEM-VLB100 [2]. When agents known to reverse mdr were added to these cell lines, the rate of fluo-3 accumulation increased in CEM-VLB100 but not in CEM-CCRF. Fluo-3 uptake was used as a functional assay for Pgp and was found to be a more rapid and sensitive assay than the accumulation of doxorubicin (dox).

Pgp is commonly expressed in B-cell chronic lymphocytic leukaemia (B-CLL). This is not necessarily the consequence of exposure to cytotoxic drugs which select for Pgp expression, as Pgp is often detected in patients with B-CLL at presentation [3, 4]. In order to clarify the functional status of Pgp in B-CLL we have studied 37 cases of B-CLL not clinically exposed to the cytotoxic drugs which select for Pgp expression. Pgp expression was determined by histochemical and flow cytometric methods using several monoclonal antibodies. The functional status of Pgp was assayed by flow cytometry utilising the capacity of verapamil to increase the accumulation of dox and the uptake of the acetoxymethyl (AM) ester of the fluorescein analogue fluo-3. This is the first reported application of the fluo-3 assay in clinical specimens.

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## METHODS AND MATERIALS

### *Clinical specimens*

Peripheral blood samples were collected from 38 patients with CLL. 37 patients had B-CLL diagnosed in the presence of a blood lymphocyte count greater than $5.0 \times 10^9$/l. These cells from patients with B-CLL expressed a surface pan-B cell (CD19) marker, were CD5-positive, clonal for immunoglobulin (Ig) light chain and displayed weak surface fluorescence with anti-human globulin. One individual with T-cell CLL was included as a control. The disease was staged according to Rai [5].

The patients had either received no treatment or glucocorticoids and chlorambucil. No patients had been given vinca alkaloids or anthracyclines.

### *Sample preparation*

Anticoagulated (lithium heparin 125 U/10 ml) venous blood (30 ml) was collected from each patient. Following dilution 1:2 in RPMI 1640 (Gibco Labs)/10% fetal calf serum (fcs) (Commonwealth Serum Laboratories) the blood was overlayed on Ficoll-Paque (Pharmacia) and centrifuged at 400 *g* at room temperature for 20 min. The mononuclear cell band at the Ficoll-Paque/plasma interface was isolated and the cells were washed three times in RPMI 1640/10% fcs prior to testing.

### *Analysis of mdr1 mRNA*

Guanidine isothiocyanate lysates were prepared from $5–8 \times 10^7$ mononuclear cells and total RNA isolated by ultra centrifugation on a CsCl gradient. Ten micrograms of RNA per slot was immobilised on Immobilon N membrane (Millipore) using a Bio-Rad slot blot apparatus. As a control, 1:100 dilutions of each of the RNA preparations were also loaded on slots and hybridised with a probe for 18S ribosomal RNA (rRNA). The pHDR5a probe, a 1.4 Kb cDNA for mdr1 [6], was $^{32}$P-labelled using a random primer labelling method. Membranes were hybridised and washed at 50°C. Membranes were exposed to pre-flashed film and levels of hybridisation were quantitated

using a Pharmacia LKB ultrascan XL laser densitometer and Gelscan XL software package.

*Analysis of mdr1 DNA amplification*

Cell lysates were incubated with 100 μg/ml proteinase K (IBI) for 3 h at 50°C. Following standard phenol DNA extraction, 10 μg of genomic DNA was digested overnight at 37°C with 50 U HindIII and EcoR1. Digested DNA was applied to an 0.8% agarose gel for size fractionation. The DNA was then transferred to a nylon Hybond-N filter (Amersham) for hybridisation with a $^{32}$P-labelled pHDR5a probe. Membranes were exposed to X-ray film at −70°C for 24 h and hybridisation levels were quantified using a laser densitometer.

*Fluo-3 and dox accumulation*

Accumulation profiles of dox (Farmitalia) and fluo-3 acetomethoxy ester (fluo-3 AM, Molecular Probes) were performed within 4 h of sample collection. Flow cytometry with fluo-3 and dox was performed in a FACScan flow cytometer (Becton Dickinson) with modifications to permit stirring and warming of the cells to 37°C. Kinetic software (Chronys, Becton Dickinson) was used to analyse changes in fluorescence against time. All analyses were performed on single viable cells.

To investigate fluo-3 accumulation, 6 μmol/l fluo-3 AM in dimethysulphoxide (DMSO) was added to prewarmed cells at an initial cell concentration of 5 × 10$^6$/ml in RPMI 1640/10% fcs. Final DMSO concentration was kept below 0.3%. Continuous data collection began immediately at rates of 200–1000 cells/s. Fluo-3 fluorescence was collected on a linear scale at 530 nm (bandwidth 30 nm) and changes in mean channel fluorescence (MCF) were plotted against time.

Dox accumulation was measured using a method adapted from Herweijer *et al.* [7]. Dox (6 μmol/l) in RPMI 1640/10% fcs was added to prewarmed cells and discontinuous data collection begun immediately for up to 210 min. The intervals between data collection points ranged from 5 to 30 min. Dox fluorescence was measured on a linear scale at 585 nm (bandwidth 42 nm) and changes in MCF were plotted against time.

The rate of accumulation of fluo-3 and dox following 20 min of exposure to 20 μmol/l verapamil (Schering) was compared to the rate of accumulation without verapamil. An increase in rate greater than 10 MCF units/min (ΔMCF/min) for fluo-3 or 10 MCF units/10 min (ΔMCF/10 min) for dox, following verapamil addition, was considered to be a functionally positive result for mdr activity. These criteria were based upon a large number of experiments in various cell lines known to be either multidrug resistant or drug sensitive [2].

*MRK-16 binding*

An aliquot of 1 × 10$^6$ CLL cells was incubated with 2.5 units of neuraminidase (nmd, Sigma) at 37°C for 30 min and then washed three times with RPMI 1640/10% fcs. Untreated and nmd-treated lymphocytes were incubated for 30 min with either 5 μg/ml IgG2a (Becton Dickinson), an isotype-matched monoclonal antibody control or 5 μg/ml MRK-16 monoclonal antibody (a gift from Prof. T. Tsuruo, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo). Cells were washed three times with RPMI 1640/10% fcs and then incubated with 2 μl of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin antibody (Becton Dickinson). Antibody labelling was determined on a FACScan flow cytometer. Cases with more than 5% of cells positive relative to the isotype matched monoclonal antibody control were recorded as positive for MRK-16 labelling.

*JSB-1 binding*

Two cytospins of peripheral blood lymphocytes from each patient were stored at −20°C. Prior to testing, the cytospins were allowed to come to room temperature and then fixed in acetone for 10 min. After washing twice in Tris-buffered saline (TBS) for 3 min, one cytospin was incubated with a 1/10 dilution of JSB-1 monoclonal antibody (Sanbio) for 1 h at room temperature. The cytospins were washed three times in TBS and antibody labelling was detected and compared to unlabelled controls using a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [8]. APAAP staining on coded cytospins was assessed independently by two assessors whose performance was verified by the use of coded cytopsins prepared from cell lines known to be either multidrug resistant or drug sensitive.

*Statistics*

Sensitivity and specificity were determined for the fluo-3 and dox assays by comparison with Pgp determined with MRK-16. Estimates of sensitivity and specificity are given by sensitivity = Tp/Tp+Fn × 100, specificity = Tn/Fp+Tn × 100 where Tp = true positive, Fp = false positive, Fn = false negative and Tn = true negative. Estimates from the assays were compared by McNemar's exact test for differences between proportions [9].

## RESULTS

37 patients with a median age of 70 years (range 50–84) were tested. The white cell count ranged from 8.8–659 × 10$^9$/l with a median of 32 × 10$^9$/l. Patients were either untreated or had received prednisolone and chlorambucil.

29 (78%) patients were positive for mdr using the fluo-3 assay and 22 (59%) patients were positive for mdr with the dox assay (Table 1). 34 (92%) patients with B-CLL were positive for Pgp using MRK-16 labelling (Table 1). Thirty-one (84%) samples were positive for MRK-16 labelling without treatment with neuraminidase (data not shown). JSB-1 detected Pgp expression in 52% of 29 cases tested. Amplification of the mdr1 gene was not observed (data not shown). Compared to the drug-sensitive cell line CEM-CCRF, only 4 of the 27 (15%) patients tested showed an increase in mdr1 mRNA. Lymphocytes from these 4 patients expressed the mdr phenotype using the dox and fluo-3 assays (data not shown). Cells obtained from a patient with T-CLL were negative for all assays of mdr (data not shown).

28 (82%) of the 34 cases in which the MRK-16 assay was positive were also positive using the fluo-3 assay (Fig. 1a, Table 1) compared to 56% (19 cases) using the dox assay (Fig. 1b, Table 1). Thus, the sensitivities of the fluo-3 and dox assays were 82 and 56%, respectively. The difference between the two estimates of sensitivity is statistically significant ($P = 0.004$) and 95% confidence intervals for true sensitivity were 69 and 95% for the fluo-3 and 39 and 72% for the dox assay.

The estimated specificities of the fluo-3 and dox assays are 67% (2/3) and 0% (0/3), respectively. However, due to the high rate of Pgp expression in CLL, little confidence can be attached to these estimates of specificity and the difference is not statistically significant ($P = 0.50$).

14 out of 28 cases positive in the MRK-16 assay were positive for JSB-1 labelling and 3 out of 25 cases that were MRK-16-positive had increased levels of mdr1 mRNA. When compared to MRK-16 labelling, the estimated sensitivity of JSB-1 labelling and mdr1 mRNA slot-blot analysis was 50 and 12%, respectively. Both of these assays were significantly less sensitive than the fluo-3 assay ($P = 0.0064$ and $P < 0.0001$, respectively).

*Table 1. Mdr assay results using fluo-3, dox accumulation and MRK-16 labelling in lymphocytes from 37 patients with B-CLL*

| Rai stage | n | Mrk16-positive Fluo-3 + Dox + | Mrk16-positive Fluo-3 + Dox − | Mrk16-positive Fluo-3 − Dox + | Mrk16-positive Fluo-3 − Dox − | Mrk16-negative Fluo-3 + Dox + | Mrk16-negative Fluo-3 + Dox − | Mrk16-negative Fluo-3 − Dox + | Mrk16-negative Fluo-3 − Dox − |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 13 | 5 | 5 | 0 | 2 | 1 | 0 | 0 | 0 |
| I | 4 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| II | 8 | 4 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| III | 5 | 2 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| IV | 7 | 5 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| All stages | 37 | 19 | 9 | 0 | 6 | 1 | 0 | 2 | 0 |

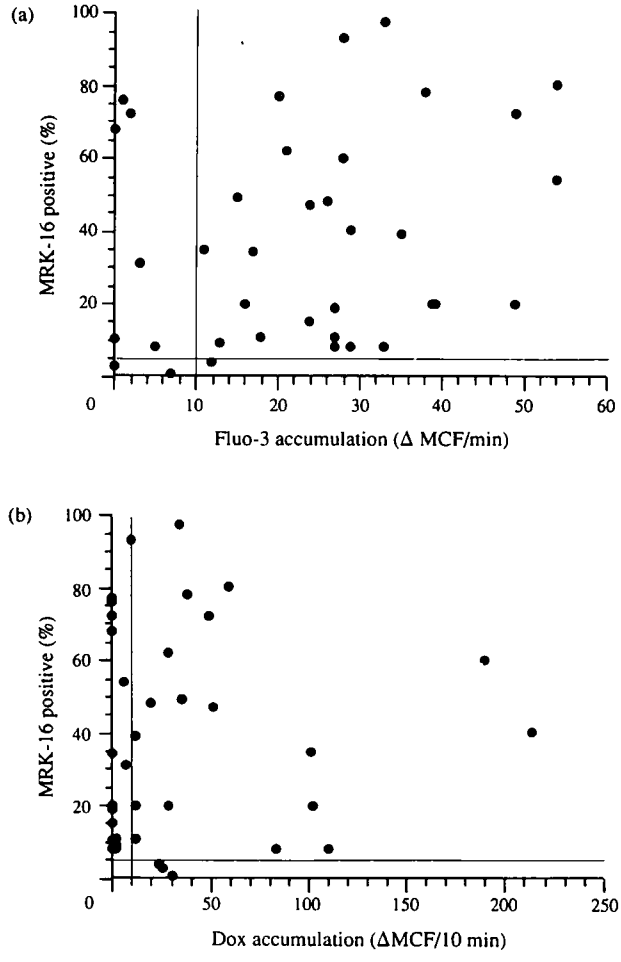


**Fig. 1. Increase in rate of fluo-3 (a) or dox (b) accumulation after addition of 20 μmol/l verapamil in lymphocytes from patients with CLL vs. the percentage of lymphocytes positive for MRK-16 monoclonal antibody labelling relative to an antibody isotype-matched control. These figures represent the maximal value for MRK-16 labelling of lymphocytes, irrespective of neuraminidase treatment. The intersecting lines represent the minimum value for a positive result in each assay.**

In this study, serial fluo-3 assays on individual patients were reproducible and the assay did not decrease in sensitivity with the maintenance of clinical samples at room temperature over 6 h (data not shown).

## DISCUSSION

The calcium-sensitive fluorophore fluo-3 has been shown to accumulate more rapidly in drug-sensitive than multidrug-resistant cell lines [2]. We have now applied this functional assay of Pgp expression to patients with CLL and report excellent concordance with standard assays of Pgp expression.

In 92% of 37 patients B-CLL cells were positive with MRK-16 labelling. This compares with previous studies using MRK-16 for Pgp detection which reported a detection rate of 50 [3] and 62% [10] using flow cytometric assays. In contrast to the report of Cumber [1], the rate of positivity was not consistently enhanced by neuraminidase. When compared with the MRK-16 labelling, the fluo-3 assay had a sensitivity of 82%, significantly greater than a sensitivity of 56% for the dox assay ($P$ = 0.004). In addition, the fluo-3 assay represented a more rapid measurement of mdr than dox accumulation.

Caution is necessary in interpreting these data as specificity could not be compared between assays because of the high proportion of positive results in this patient group. However, Campos [11] also found a poor correlation between Pgp expression measured using MRK-16 labelling, and a functional assay based upon daunorubicin retention in leukaemic cells from patients with acute myeloid leukaemia. This finding is consistent with our experience which also demonstrated the poor sensitivity of anthracycline-based functional assays.

JSB-1 labelling and the detection of mdr1 mRNA using a slot blot technique was less sensitive than the MRK-16 assay in this study. Using an RNAse protection assay, Herweijer [4] found that 100% of 17 patients with CLL expressed both mdr1 and mdr3. In this study, 1 case which was positive by mdr1 mRNA slot-blot analysis but negative in the MRK-16 assay, was positive with the fluo-3 and dox assays.

Haber [12] recently reviewed the current evidence for the role of mdr in leukaemia and supported the clinical need for a routine Pgp assay. In acute leukaemia [12], neuroblastoma [13] and osteogenic sarcoma [14], Pgp expression appeared to be an adverse prognostic factor. Haber defined the ideal Pgp assay as one which should distinguish expression of mdr1 from mdr3, detect small subpopulations of positive cells and be reproducible. The fluo-3 assay appears to meet these criteria. It only detects the functional protein product of the mdr1 gene and is rapid, simple and sensitive. In addition, flow cytometry confers the capacity to identify small subpopulations of cells. Herzog [15] suggested that the most sensitive assay of mdr in colon carcinoma cell lines was the use of polymerase chain reaction (PCR) analysis to measure mdr1 mRNA. However, there may not always be good correlation between mdr1 mRNA expression and functional Pgp expression at the cell surface. Furthermore, such an assay could show false positivity in situations where the clinical samples contain non-malignant cells which normally express mdr1 mRNA.

These studies reporting enhanced Pgp expression in CLL

raise the question of the significance of *de novo* expression of this glycoprotein in this patient group. In some haematological malignancies such as myeloma, Pgp expression is usually detected following drug treatment. The most obvious question these data raise is whether the finding of intrinsic Pgp expression in CLL is associated with clinical drug resistance?

CLL is often an indolent disorder. In the early stages it is either not treated or treated with glucocorticoids and an alkylating agent, drugs which albeit quite effective are not known to select for mdr in cell lines. Desai [16] found no response of CLL to vincristine as a single agent. Keating [17] showed that a regimen which included prednisolone and an alkylating agent but also contained vincristine and doxorubicin (POACH) showed no advantage over simple prednisolone and chlorambucil in previously untreated CLL. Conversely, the French Co-operative Group [18] showed an advantage of CHOP over CVP in advanced CLL, and ECOG reported a response rate of 30–40% with VAD in advanced CLL [19]. However, dexamethasone alone is an effective agent in CLL and it has been suggested that the drug resistance phenotype may confer dexamethasone sensitivity [20].

In general, the clinical significance of the response of CLL to combinations of drugs is difficult to interpret. Multidrug resistance may simply modify drug response in an environment in which multiple mechanisms of drug resistance may be in operation. In addition, drugs such as the vinca alkaloids may act as pump modulators for other cytotoxics (normally handled by Pgp) that are present in the same combination.

Weisenthal [21] examined the drug sensitivity of patients with CLL using the *in vitro* differential staining toxicity assay (DISC). In 18 out of 30 patients the study demonstrated drug resistance. In these individuals drugs which included modulators of Pgp enhanced *in vitro* toxicity to vincristine. Perri also examined drug sensitivity using the DISC assay in 22 patients with B-CLL [22, 23]. He found a high degree of *in vitro* drug resistance which related poorly to Pgp expression but closely to the presence of mdr1 mRNA detected by slot blot analysis. However, Shustic [24] found no relation between the detection of mdr1 mRNA and subsequent clinical disease response. Further studies are obviously required to clarify the biological and clinical significance of Pgp expression in CLL.

In conclusion, whilst there was relatively poor correlation between the degrees of positivity in the fluo-3 assay and the detection of Pgp using the monoclonal antibody MRK-16, there was excellent concordance between the two assays. The significance of these findings for disease progression or responsiveness for drugs implicated in multidrug resistance is uncertain and needs to be clarified in prospective studies.


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