Pergamon

European Journal of Cancer Vol. 32A, No. 6, pp. 1024–1033, 1996
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Printed in Great Britain
0959-8049/96 $15.00+0.00

S0959-8049(96)00045-7

How to Probe Clinical Tumour Samples for P-glycoprotein and Multidrug Resistance-associated Protein

H.J. Broxterman, J. Lankelma and H.M. Pinedo

Department of Medical Oncology, BR 232, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands

INTRODUCTION

ONE FACTOR which supposedly limits the effectiveness of chemotherapy in cancer treatment is a reduced net uptake of cytostatic drug into the tumour cells, caused by the over-expression of plasma membrane drug transporters, leading to a multidrug resistant (MDR) phenotype. It has now been shown that two drug transporter proteins affect the net uptake of lipophilic natural product agents by human cancer cells. These proteins are P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP). With the availability of gene probes and monoclonal antibodies against these proteins, the incidence of Pgp and MRP in clinical tumour specimens has been determined [1–4]. In general, results from many of the published studies on Pgp distribution in tissues and tumours are qualitatively quite similar, showing prominent Pgp expression in certain tissues (adrenal gland, liver, epithelium of gastrointestinal tract) and tumours (differentiated renal and colon cancers), but low, heterogeneous or undetectable Pgp in other tissues and tumours [1–3]. As far as MRP expression is concerned, the available probes and antibodies have been used to study its general distribution pattern [4–8].

However, what the quantitative role of Pgp (and MRP) is in clinical drug resistance is unknown, with current methodology giving irreproducible and inadequate answers [9–11]. Possible reasons for this and possible improvements in MDR detection methodology are the topic of this paper.

GENERAL CONSIDERATIONS ON Pgp AND MRP DETECTION

The primary point to consider is the sensitivity of the methodology to be used. It has now been established in a number of investigations that Pgp levels in clinical material are low, especially in those cancer types which are responsive to chemotherapy and in which expression of resistance markers may have some impact on clinical decisions. Cancers with relatively high Pgp expression, such as adrenocortical cancer and differentiated renal cell or colon cancers, are intrinsically resistant to all MDR drugs and, therefore, analysis of such markers is not expected to be helpful in the clinic [12]. In contrast, in diseases such as leukaemia, myeloma, lymphomas and solid tumours, such as breast cancer and ovarian cancer, knowledge of Pgp, MRP or other resistance markers, such as lung resistance protein/major vault protein (LRP/MVP) (see Izquierdo and colleagues, pages 979–984), glutathione status or topoisomerase II activity, may be helpful in certain patients. For instance, one relatively recent issue is the role of *MDR1*/Pgp in ovarian cancer, now that treatment with paclitaxel is being increasingly used for the treatment of this disease [13]. Since paclitaxel is very efficiently transported by Pgp, it could be that low levels of Pgp expression, such as those occurring in ovarian cancer, might be of critical importance. If it is true that even very low levels of Pgp expression are predictive for chemotherapy outcome, as suggested for ovarian cancer and small cell lung cancer (samples were considered positive with a value of *MDR*1 mRNA expression between KB3-1 "sensitive" and less than 2-fold resistance KB8 cells), then probably only polymerase chain reaction (PCR) techniques are suitable for such studies [14]. For MRP, such studies have not yet been performed, but it seems clear that MRP is constitutively expressed at a low level in almost every cell, with relatively high expression in only a few tissues, such as bronchi, heart, muscle and adrenal cortex [7], and tumours (lymphocytic leukaemia, oesophagus squamous cell carcinoma, non-small cell lung carcinoma) [4, 7]. This implies that methods of detecting MRP must not only be sensitive (i.e. detect low levels of MRP expression), but must also be able to quantitate low levels of expression.

In this paper, we will discuss some methodological considerations of importance for reliable Pgp and MRP detection in clinical tumour samples. Our main focus will be on sensitivity and quantitation, since it is likely that we shall have to aim at detection and discrimination of low levels of expression which differ by a factor of two or less. While a routinely applicable method needs to be specific and quantitative, it should be noted that, to our knowledge, no published MDR detection methods have yet been tested rigorously for their accuracy and reproducibility (day-to-day variation of real samples, etc.), as is required for validating analytical chemical procedures. In fact, the failure to do so before including a test in a clinical study might very well be the cause of much confusion and, more disturbingly, of the possibility that wrong conclusions are drawn from such studies.

Correspondence to H.J. Broxterman

mRNA MEASUREMENTS

Determination of *MDR*1 mRNA expression in many human tumour samples requires a method with the sensitivity of the reverse transcriptase PCR (RT-PCR) [3]. Another method with a high sensitivity, provided that sufficient material is available, is the RNase protection assay [15]. It has been estimated that with an RNase protection assay, approximately 1×10^6 target transcripts can be detected, in contrast to approximately 1×10^7 with Northern blotting [16].

RT-PCR

Measurement of *MDR*1 or *MRP* mRNA (over)expression in clinical tumour samples requires appropriate controls for recovery of RNA and quantitation of the signals obtained. The choice of probes and enzyme concentrations (RNase protection), primers and reaction conditions (RT-PCR) and a detection system may be critical and may be different per tumour type (e.g. choice of control gene γ-actin, β-actin, β_2-microglobulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH); recent practical laboratory guides will give the reader information on all the general theoretical and practical considerations). However, it should be borne in mind that, because RT-PCR is a very sensitive, quantitative method for determining gene expression it requires highly standardised reaction conditions for each sample and control. This makes the method quite laborious, since serial dilution of cDNAs to ensure that amplification is in the exponential range, is crucial [16,17].

As an example of the importance of quality control of the methodology, the results of three studies which measured *MDR*1 mRNA expression in human blood cell lineages have been compared [18–20]. In general, all three papers agree that the highest Pgp expression is detected in $CD56^+$ natural killer (NK) cells and $CD8^+$ T suppressor cells, that Pgp is intermediate in $CD4^+$ T helper cells and absent in $CD14^+$ monocytes, based on Pgp surface expression or dye efflux. Two of the papers used the 8226dox6 myeloma cell line as a control. In one study, which used an RT-PCR method with an *in vitro* synthesised RNA standard, the highest *MDR*1-expressing ($CD56^+$) cells had a value of 50% that of 8226dox6 [20]. The other study, which used an RT-PCR method with β_2-microglobulin primers as a control, found an *MDR*1 mRNA expression in $CD8^+$ and $CD56^+$ cells of more than 6-fold that of 8226dox6 and 2-fold that of 8226dox40, a myeloma cell line which was more than 100-fold resistant to doxorubicin (Dox) [19]. Such a high expression of Pgp in unselected cells is highly unlikely and, if real, would have enormous implications for chemotherapy. More likely, these results cannot be regarded as quantitative and cannot be compared with other studies. Again, it is important to realise that this has to be seen in the context of detecting a factor of 1.5 or 2 difference in Pgp/*MDR*1 expression at a level considerably lower than the 32-fold doxorubicin-resistant 8226dox6 cells [20].

RNase protection

We have used RNase protection in the past to show that the low *MDR*1 mRNA expression in parental SW-1573 lung cancer cells is actually downregulated upon induction of the non-Pgp MDR phenotype in this cell line. The expression becomes undetectable by RNase protection or by RT-PCR in SW-1573/2R50 and 2R120 non-Pgp MDR cells [21]. Subsequently, we have studied *MDR*1 and *MRP* mRNA expression in acute myeloid leukaemias (AML) with an RNase protection assay, quantitated by densitometric scanning of the labelled bands, corresponding to *MDR*1 or *MRP* mRNA, and divided by the γ-actin band, used to control for RNA loading. We did not find a correlation between the values found for Pgp expression measured by flow cytometric labelling of viable cells with monoclonal antibody MRK-16 (see below) and *MDR*1 mRNA in these samples (Figure 1) [22]. Although the discussion about the appropriate control (γ-actin, β-actin, β_2-microglobulin or GAPDH) is partly the same as for RT-PCR, there is at least one plausible biological reason for this discrepancy. *MDR*1 mRNA expression levels may vary 200-fold between different acute myeloid leukaemias (AMLs) with some AMLs having very low expression (Figure 1). This means that a low percentange of contaminating normal *MDR1*-expressing cells (e.g. T cells) may have a significant increasing effect on low *MDR*1-expressing AMLs. Contaminating normal lymphocytes may also lower the *MDR*1 mRNA signal of high expressers. In both cases, these problems may be less when using a flow cytometric Pgp assay, because a low percentage (e.g. 1%) of relatively high Pgp expressers will be separate from the main peak. Alternatively, to a certain extent normal cells can be gated out by scatter characteristics.

Another reason to be more confident of a flow cytometric Pgp protein assay is that we have seen examples of *MDR*1 mRNA-expressing cell lines, without detectable Pgp expression or function (see SW-1573 cell line in Figure 1).

As far as *MRP* expression is concerned, the above considerations might be less relevant since all normal peripheral blood cells, regardless of cell lineage, express a similar basal level of *MRP* mRNA [23] and, accordingly, the variation in its expression in AMLs seems to be less (about a factor 10) than for *MDR*1 mRNA expression [22]. This allows less interference with the resulting tumour signal by small subpopulations of contaminating normal cells. Of course, the loss of any insight into the heterogeneity of expression within the tumour cell population, when assayed by RT-PCR of mRNA from a cell homogenate, remains a drawback of this technique for *MRP*, as well as for *MDR*1 mRNA.

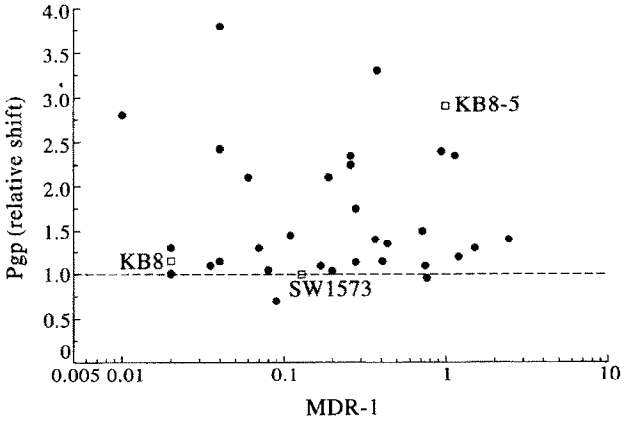

Figure 1. Correlation between *MDR*1 mRNA and P-glycoprotein (Pgp) expression in acute myeloid leukaemia (AML) and cell lines. *MDR*1 mRNA was measured with RNase protection assay. Pgp was measured with MRK16 (5 μg/ml, 60 min, room temperature) and FITC-labelled second antibody and quantified on a FACSTAR (details in [22]).

In situ *hybridisation*

A resolution of cellular heterogeneity at the mRNA level can be achieved by *in situ* hybridisation. It has been shown by this technique that, in lung cancers, *MRP* expression is particularly strong in lymphocyte infiltrate and in tissue-invading tumour cells [24]. Thus, while useful in certain cases, *in situ* hybridisation has not gained a place among current MDR screening protocols. The reasons for this might be that it is a rather laborious technique, for which the sensitivity in comparison to other methods with cellular resolution (i.e. immunohistochemistry) has not yet been documented in clinical samples. One study has compared *MDR*1 *in situ* hybridisation with immunocytochemistry, immunoblotting, flow cytometry and RT-PCR in resistant cell lines (lowest resistance factor of approximately 20–30 for vincristine), and it appeared that quantification of the lower signals against the background was not satisfactory [25]. Reasonably satisfactory results were reported in 36 "freshly established cell lines" from childhood acute lymphoblastic leukaemia samples (80% concordance between RT-PCR and *in situ* hybridisation). However, discordant results have been reported with MRK16 staining [26].

PROTEIN DETECTION

The techniques used for Pgp detection are immunoblotting, immunohisto/cytochemistry and flow cytometry. From all the techniques used for *MDR*1/Pgp detection, these methods based on monoclonal antibodies are the most widely used. The reason for this is undoubtedly their relatively easy application for routine pathological screening. Accordingly, many papers have been published over the last 10 years which use these immunostaining methods to measure Pgp in archival clinical tumour specimens. Prospective studies in which tumour samples are prepared according to controlled, optimised protocols for fixation, antigen preservation or retrieval, reaction conditions etc., are still very scare.

Western immunoblotting

Western blotting is not a method of first choice, because evidently no distinction can be made between tumour or stromal cells which is an important issue in MDR analyses. In addition, the relatively laborious methodology does not facilitate routine application. Nevertheless, since by Western blot analysis, a positive identification of the molecular weight of Pgp or MRP can be obtained, which may help in the elucidation of crossreactions in suspected cases, it may help in confirming the presence of these proteins. One has to be aware of the possibility of falsely negative or positive results which may be caused by proteolytic breakdown or cross-reaction of the antibodies. The most used monoclonal antibody for Pgp is C219, which may crossreact with the *MDR*3-encoded Pgp, for instance in B-cell prolymphocytic leukaemia [27]. Less crossreaction with *MDR*3/Pgp has been reported for C494 and JSB-1 antibodies [28]. Similarly, Western immunoblotting may help in identifying proteins crossreactive with monoclonal antibodies against MRP, such as MRPr1 and MRPm6 [29]. With the advent of enhanced chemiluminescence (ECL) detection, the methodology now seems to be sensitive enough to detect KB8 level of Pgp without the need for ^{125}I-based detection [27]. In summary, in selected cases, immunoblotting may be applied to confirm the presence of Pgp or MRP.

Immunohisto/cytochemistry

The single most widely adopted technique for Pgp detection in clinical tumour specimens is immunohistochemical staining. To date, two laboratories have published immunocytochemical data, suggesting that (extremely) low Pgp levels can be detected by immunohisto/cytochemical staining. A study by Grogan and coworkers [30] suggests that immunocytochemistry, when performed under stringently controlled conditions, can be a reliable test for Pgp expression in myeloma and may come "within the realm of everyday hospital laboratory expertise". However, it must be noted that the lowest resistant cell line used in this study to define the limit of detection was the 8226/dox4 myeloma cell line. This cell line has an MDR phenotype which is still high compared to clinical samples. We showed that 8226/dox4 cells had a relatively low nuclear/cytoplasmic doxorubicin ratio (2.2 compared to 3.9 for 8226S cells; see below), a functional MDR parameter [31], and we estimated the number of Pgp molecules per 8226/dox4 cell to be in the order of 200000 similar to that of KB8-5 cells (unpublished data). A lower level of detection is still needed for many tumour types.

In order to measure such low levels of Pgp expression in paraffin-embedded, formalin-fixed, solid tumour specimens, a four-layer immunoperoxidase-based "sandwich" technique has been used by Chan and colleagues [32] In a retrospective study, it was found that Pgp was highly predictive for prognosis in childhood rhabdomyosarcoma and neuroblastoma [32]. Unfortunately, these authors did not use the same control cell lines as other investigators and, therefore, we cannot compare the sensitivity of their method with the quantitative PCR in KB cell lines [3]. One paper claiming to apply the method of Chan and colleagues could not detect the highly resistant KB8-5 cells [33]. Another team claims to have developed an improved multilayer peroxidase technique [34]. They use overnight staining with the primary antibody JSB-1, which, they say, performs better than C219 in paraffin sections because its epitope appears to be more resistant to tissue processing [34]. However, KB8-5 cells again seem to be in the margin of detection. In the experience of other investigators, who use conventional peroxidase-based immunostaining, JSB-1 and other antibodies give an unsatisfactory, non-specific background in paraffin-embedded material [35]. Therefore, as a conclusion from the published immunoperoxidase-based studies, Pgp detection above a certain expression level, which probably varies per study, is possible [36], but the general applicability of a sensitive Pgp detection method has yet to be proven. From this, it may be inferred that the expression level and accordingly the role of Pgp in many low-level Pgp-expressing or initially chemotherapy-sensitive tumour types has not yet been adequately studied. There is, for example, no published study using a highly sensitive, quality controlled immunoperoxidase method examining the expression of Pgp in breast cancer. This may even hold for frozen sections which seem to be relatively accessible to Pgp staining [37–39].

An alkaline phosphatase anti-alkaline phosphatase (APAAP) version of the four-layer technique has been published, which can be used for staining myeloblasts in which endogenous peroxidase is present [40]. With this technique, 8226/dox6 cells are scored as having a 3+ plasma membrane staining for Pgp. We have used a double APAAP staining for acetone-fixed AML samples (with 15 μg/ml JSB-1 or C219 primary antibody incubation for 1 h at room temperature) and could only stain those AMLs with the highest Pgp expression,

which are between the expression of KB8 and KB8-5 cells as determined with flow cytometry. Unfortunately, KB cells have a high background with the APAAP technique and could not be used as controls.

In contrast to immunocytochemical Pgp detection, the detection of MRP in tumour cell lines with currently available monoclonal antibodies (MRPr1, MRPm6) [29], using the same methodology, is very sensitive. The MRPr1, MRPm5 and MRPm6 antibodies all perform better on fomaldehyde-fixed, paraffin-embedded than on frozen sections, probably because they recognise non-conformational epitopes [7]. We used MRPr1 on acetone-fixed cytospins of a series of small cell lung cancer cell lines with increasing drug resistance factor [41] to test the sensitivity of the double APAAP method for MRP detection. These results (see Table 1) showed that plasma membrane-associated MRP could be detected in the parent GLC_4 cells in accordance with the finding that a baseline *MRP* mRNA expression is present in all tissues. Thus, the major difficulty will be to make a reliable distinction between MRP staining of GLC_4 ("sensitive") cells and that of 2-fold resistant cells. Preliminary results of the staining of AML samples showed little variation, and possibly an optical image analysis system, such as used by Grogan and associates [30], would be helpful. In addition, preliminary data have suggested that MRP might be intracellularly located in various normal tissues, but located in mainly plasma membranes in tumour cell lines and clinical tumour samples [7]. Since, at present, the role of intracellular MRP in mediating drug transport is unclear, it seems imperative to document unambiguously whether MRP is on the plasma or intracellular membranes.

In conclusion, it is clear that reliable, sensitive detection of Pgp with immunohisto/cytochemical techniques in clinical material requires a commitment and investment in personnel and time, which is usually not available in a routine pathology setting. Interestingly, the same problems in variation of immunocytochemical analysis are encountered in other fields, and interlaboratory control studies are performed in order to analyse the influence of variables (fixation, primary antibody, RAM and APAAP dilution, incubation times and temperatures, counter stains, etc.) [42]. Such quality control studies are just beginning to be performed in the MDR field (results of Memphis MDR detection workshop, Beck and colleagues [35]). In addition, there is a clear place for anti-Pgp antibodies with higher affinity for Pgp than the presently available antibodies, which would allow us to use lower concentrations of antibody with lower background staining (see also at flow cytometry) [43].

Table 1. MRP detection in GLC_4 sublines

Cell line*	Dox resistance*	APAAP†	Flow cytometry‡	Flow cytometry§
GLC_4	1	+/−	1.6	2.7–1.8
GLC_4-ADR_2	2	+/− +	1.9	n.d.–2.6
GLC_4-ADRpr	11	++	2.3	4.0–4.7
GLC_4-ADR_{150}	150	++++	7.4	13.6–21.1

APAAP, alkaline phosphatase anti-alkaline phosphatase; Dox, doxorubicin; n.d., not determined.

**MRP* mRNA expression and resistance factors in these cell lines are described in [41]. †Procedure "double APAAP" immunocytochemistry: ice-cold acetone fixation (10 min); 20 min pre-incubation with blocking buffer (10% rabbit serum + 1% BSA); 60 min primary antibody MRPr1 (1.7 μg/ml; room temperature); 30 min rabbit-anti-rat (RAR, Dakopatts 1:50, room temperature); 30 min APAAP (rat; Dakopatts 1:25, room temperature); repeat RAR and APAAP step; 30 min alkaline phosphatase substrate (room temperature, dark); counterstain: haematoxylin. ‡Flow cytometric detection of MRP in cells permeabilised for 10 min with 10% lysing solution G (v/v) (Becton Dickinson) (from [50]). §Flow cytometric detection of MRP in cells permeabilised for 5–10 s with ice-cold 2% formalin solution in acetone. In both cases, labelling with MRPr1 (1.7 μg/ml, 60 min, room temperature) and rabbit anti-rat FITC (Dakopatts, 1:100, 45 min, room temperature, dark). Data are ratios of mean fluorescence with MRPr1 divided by mean fluorescence of isotype control or PBS (two separate experiments on different flow cytometers).

Flow cytometry

To address the issue of cellular heterogeneity, which is likely to play a role in the determination of Pgp or MRP in many clinical tumour samples, flow cytometry seems to be particularly suited. Firstly, the problems of Pgp flow cytometric assays have been mainly related to their sensitivity, quantitation, standardisation and quality control, a typical example of the transition from classical pathology to analytical cellular, quantitative pathology [11]. Secondly, flow cytometry seems to be limited mainly to haematological malignancies, until procedures to obtain single cell intact tumour cell populations by disaggregation of solid tumours are more efficient. This discussion will only deal with Pgp and MRP detection in haematological malignancies, since Pgp detection by flow cytometry in clinical solid tumours has not been reported.

A third consideration is that, in principle, flow cytometry allows Pgp or MRP measurement in certain subpopulations of cells (e.g. $CD34^+$ cells) or a combination with a functional drug pump assay (see below).

A vital point for flow cytometric Pgp detection is the choice of antibody. All available evidence strongly supports the use of antibodies recognising a cell surface epitope of Pgp (MRK16, 4E3, UIC2 or others) instead of those directed against an intracellular epitope (C219, C494, JSB-1 or others) [35]. The former can be used to stain intact cells, which eliminates the need for permeabilisation of the cells, leading to a possible decrease in the signal-to-noise ratio [44]. Moreover, the use of intact, viable cells allows for the combined assessment of Pgp and its function by dual parameter flow cytometry. Unfortunately, monoclonal antibodies against cell surface epitopes of MRP have not yet been described.

One disadvantage of the use of antibodies against cell surface epitopes might be epitope masking by sialic acid residues, which has been reported for chronic lymphocytic leukaemia blast cells [45]. Since we did not find any effect of neuraminidase treatment of AML samples on staining with MRK-16 [46], it may have to do with suboptimal concentrations and staining conditions, used in the former study (2.5 μg/ml, 30 min at 0°C). Our present conditions are 25 μg/ml MRK-16, 60 min at room temperature; saturation was only approached when 100 μg/ml was used [46]. Thus, because of the low affinity of antibodies, such as MRK-16 or UIC2, in combination with the low Pgp expression in clinical tumour samples and normal blood cells (we have estimated that KB8 cells have in the order of 20000 Pgp molecules and KB8-5 and 8226/dox4 cells in the order of 200000 using the Qifikit^R (Biocytex, Marseille, France)]) [47], a high concentration of isotype control antibody is applied, which is presumed to

produce similar background fluorescence to the test antibody [26]. However, this is not the case with all brands or batches [46]. Alternatively, the use of a high concentration of secondary antibody has been advocated in order to enable the detection of Pgp expression in bone marrow cells [48]. These examples again show that it is essential to perform quality control experiments using low level Pgp expressing cell lines, such as KB8 cells [3] and to use a combination with other techniques, such as functional assays.

An important point is that, because the expression level of Pgp in clinical haematological samples is usually around the KB8 level, a considerable overlap between negative control antibody-labelled cells and test cells is obtained. Therefore, one has to express the data as a shift of the mean (or median) and not as percentage positive cells [35]. Another possibility is to use Kolmogorov–Smirnov statistics for comparison of positively stained cells with controls [35]. However, both methods of data representation showed excellent correlation [49]. Increased sensitivity of detection may be reached by using biotin–avidin–Texas RED instead of FITC labelling [49]. Irrespective of the method, one should establish the detection threshold of the adopted procedure with an appropriate control cell.

Since, as yet, no monoclonal antibodies against cell surface epitopes of MRP have been described, we have used plasma membrane permeabilisation methods in combination with the MRPr1 antibody [29] to measure MRP expression in tumour cell lines and AML samples. Table 1 shows that this procedure did not give a consistent resolution of the lowest resistant cells within the relevant range of resistance. SW-1573 (MRP) transfectant cells with a resistance factor of 3–4 were easily detected [50]. The majority of AML samples, assessed with this procedure, gave a value at or below GLC4 and SW-1573 "sensitive cells" (Broxterman and colleagues, unpublished). A factor which may lead to unsatisfactory sensitivity in detecting the lowest levels of MRP-mediated resistance is the finding that low levels of resistance may not be related to increased expression of MRP protein, but to slightly altered (increased glycosylated) MRP protein [51]. For this reason, it would be important to measure only plasma membrane MRP in intact cells. For a correlation of MRP protein with MRP function see below.

DRUG TRANSPORT OR FUNCTIONAL ASSAY

A particularly suitable opportunity for specific detection of the MDR phenotype is probing the functional drug transporter phenotype. Such assays have the clear advantage that they measure the relevant property of the MDR-related proteins, i.e. their transport capacity. Moreover, the testing of pump inhibitors may give a lead to treatment of a patient with the most effective modulator. Different techniques to probe MDR transport function will be discussed here.

Since we require a high sensitivity for the assays, only fluorescent or radiolabelled probes are discussed here. Many highly fluorescent dyes are transported by Pgp or MRP and the application of these molecules to detect the MDR-related drug transport in cancer cells may become a successful analytical application of fluorescent probes in oncology.

Flow cytometry

By far the most widely used technique to probe Pgp function is the assessment of fluorescent dye or drug accumulation or efflux by flow cytometry. This can be done by virtue of the fact that many fluorescent dyes are hydrophobic compounds, which are efficiently taken up and transported out of the cell by Pgp. In the past, the accumulation of such dyes has been used to assess other characteristics of the cell, but it is now known that their active transport has to be taken into account. Among these dyes are rhodamine 123 (R123; mitochondrial dye), Hoechst 33342 (DNA binder), BCECF-AM (pH indicator), $DiOC_2(3)$ (membrane potential indicator), Fura-2-AM and Fluo-3-AM (calcium ion indicators), calcein-AM (viability probe and cytoplasmic marker) and the anthracyclines, daunorubicin (Dnr), Dox and idarubicin (Ida) (DNA binders).

When choosing an optimal probe for the assessment of Pgp activity, a number of factors have to be considered. Theoretical requirements for an ideal MDR probe are summarised in Table 2. Essentially, these criteria refer to the probe's sensitivity, selectivity and practicability. There are no publications which have compared all the possible probes in low resistant cell lines and in clinical samples. Therefore, the following considerations are by no means exhaustive in that the single best Pgp or MRP test has not necessarily been identified yet.

Probes for Pgp functional assays. Although different studies are not comparable because of different concentrations of dyes or modulators, incubation times and inclusion of a dye efflux period in the assay, it seems to be generally accepted that R123, $DiOC_2(3)$ and calcein-AM are sensitive Pgp probes if assessed in combination with a resistance modulator such as SDZ PSC833 [48–50]. If such a modulator for assessing the difference in dye accumulation with and without Pgp activity is not used, the assays are, in general, much more difficult. Differences in absolute accumulation may be caused by small differences in, for instance, the number of mitochondria (R123), membrane potential ($DiOC_2(3)$), DNA content (Dnr), acidic cytoplasmic vesicles (Dnr) or, in general, by viability of the cells or small differences in experimental procedures. Therefore, inclusion of a modulator in the assay greatly enhances the accuracy and reliability of the assay.

Drugs should not be used at too high concentrations, to avoid possible saturation of pump capacity, especially in low Pgp-expressing cells. For instance, the saturation of Dnr transport as well as DNA binding begins to have affect above 2 μM for Pgp as well as MRP [52, 53]. Alternatively, if the number of cells is too high, the medium concentration of a drug may fall too much. In such a case, the effect of a pump inhibitor may be estimated erroneously low. This may occur more

Table 2. Theoretical requirements for an ideal multidrug resistance (MDR) probe

1. General fluorescence properties for flow cytometry (high fluorescence and no or reproducible quenching)
2. High cellular accumulation (or high distribution volume)
3. Rapid equilibration
4. High ratio of active to passive plasma membrane passage
5. Rate of plasma membrane transport determines the total loss rate of probe from the cell
6. pH-independent transmembrane transport
7. Membrane potential-independent transport
8. All properties, except MDR-related efflux, unaffected by pump inhibitors (among others, intracellular probe distribution and passive membrane transport)

readily when a very lipophilic drug, such as Ida, is used in a functional assay [54, 55].

Modulators for Pgp functional assays. The choice of modulator is also critical. The concentration of the modulator should be high enough to inhibit completely the active dye transport by Pgp, but higher concentrations may increase the risk of non-specific (not Pgp related) effects on dye accumulation. A low specificity for Pgp may even be found with commonly used modulators such as verapamil or cyclosporin A at fairly low concentrations (e.g. 8 μM verapamil [46] or 3 μM cyclosporin A [49, 56], but this also depends on the dye or drug used with the modulator [57, 58]. Based on cell line experiments [50], we studied fresh AML samples and compared the modulation of R123 and Dnr fluorescence by 2 μM SDZ PSC833 with modulation of radiolabelled Dnr or vincristine accumulation by 2 μM SDZ PSC833 or 8 μM verapamil. Based on correlation with MRK-16 staining, we concluded that in AML, SDZ PSC833 seems to be a specific modulator of the transport of all three drugs by Pgp [46]. For the choice of modulator, it should always be determined whether it influences the fluorescence yield of the probe when both are intracellulary present. It should be noted that we have seen drug pump-independent effects of PSC833 on R123 fluorescence in certain cell lines, but this did not seem to interfere significantly with the high sensitivity of the assay for Pgp function as measured by flow cytometry. Importantly, the effect of SDZ PSC833 on R123 fluorescence is highly predictive for its effects on Dnr accumulation [46].

Conditions for Pgp functional assays. It is clear that assays for an ATP-dependent plasma membrane process require conditions which allow the cells to be assayed while maintaining their metabolic and membrane integrity throughout the entire procedure. A minimal, but not sufficient requirement is that the cells exclude Trypan blue before and after the assay. ATP concentrations have to be quite high for optimal pump activity [52]. Optimally cultured, logarithmically growing, freshly harvested cells will usually be in a good condition for a functional assay. Mild trypsinisation does not seem to compromise drug efflux, but the conditions of attached growing cells may decline when stored at room temperature. Since we and others [59] have noticed a partial decrease of drug efflux function upon storage of blood samples, the use of freshly isolated leukaemic samples is recommended for a functional assay. For this reason, we also prefer short-lasting assays, instead of long duration efflux assays. Probably, a R123 or $DiOC_2$ efflux assay can be applied on correctly frozen (in liquid nitrogen) and thawed AML or normal lymphocytes [18,20,46,49]. Cells thawed after storage in liquid nitrogen are usually allowed to recover in a high protein-containing medium (20% or more fetal calf serum). The functional assays are usually carried out in medium containing a high capacity buffer (Hepes), and in the presence of 10% fetal calf serum to allow stable (pH) conditions and minimise drug binding to vials. It has to be noted that the use of (higher concentrations of) fetal calf or human serum or serum albumin profoundly influences the net effect of an added modulator because of protein binding [60] and the putative presence of endogenous modulators [61].

MRP functional assays. Although much less experience has been obtained with functional MRP assays, the same general considerations with regard to cellular conditions etc. for Pgp functional tests (see above) still hold. However, an important additional factor to be kept in mind is that the efflux of positively charged or neutral anticancer drugs or dyes (anthracyclines, vincristine, etoposide, R123) is dependent on the intracellular glutathione concentrations [6, 62–64]. Even a moderate depletion of intracellular glutathione may decrease the transport rate of these drugs by MRP [62]. Therefore, an accurate measurement of the efflux of such drugs by MRP may also require monitoring of the intracellular glutathione levels during the drug transport assay. The measurement of cellular glutathione can, in principle, be performed by flow cytometry or fluorescence microscopy, but is far from being routinely applicable. The interested reader is referred to several recent research papers on this subject [65–69]. If sufficient material is present, conventional biochemical assays for intracellular glutathione concentrations can be performed.

Since it has been shown that fluorescent organic anions, such as calcein or BCECF, are substrates for MRP, in contrast to Pgp, and since their efflux does not change after partial glutathione depletion [70, 71], they may be used for an MRP functional assay. In fact, the use of calcein-AM to load cells with calcein provided a much more sensitive assay for MRP function than, for instance, R123 efflux. Since, in combination with an organic anion modulator, such as probenecid, sulphinpyrazone or benzbromarone [70, 71], the assay is specific for MRP, even a simple, short calcein-AM accumulation instead of the need for a calcein efflux measurement seems possible. However, caution is called for because the effect of modulators on transport of the organic anion may not be predictive for their effects on (positively charged) anticancer drugs [70, 71].

As to the sensitivity of the calcein-based MRP assay, we could detect basal efflux activity in HL60 leukaemia, SW-1573 and GLC_4 parent cells, when defined as inhibition of 60 min calcein efflux by probenecid [71]. On the basis of MRP staining, these cells would all be expected to have some basal MRP activity [50] (see Tables 1 and 3). We could easily identify overexpression of MRP activity in the SW-1573 (MRP) transfected cells by using the calcein-AM/probenecid assay [71]. A Dnr/genistein assay was of similar sensitivity [50]. Since we cannot distinguish GLC_4 cells from GLC_4-ADR_2 cells (see Table 3), an even more sensitive functional MRP assay would be needed to analyse the small differences in drug efflux that might be relevant in clinical tumour samples.

Radiolabelled drug-based assays

The earliest experiments measuring Pgp efflux function made use of radiolabelled substrates. Their use in clinical detection of Pgp function has remained far behind fluorescence-based measurements, primarily because they are considered to be laborious and because the handling of radioactive materials is supposedly not compatible with routine testing [72]. Although these practical drawbacks may be partly true, depending on the local laboratory facilities, it has to be considered that radiolabelled accumulation assays have some theoretical, scientific advantages above fluorescence measurements. These assays can be performed very accurately if sufficient cells are available [60, 73, 74]. Advantageous is the fact that interference by slight differences in reaction conditions or by the added modulator of drug transport, as are frequently observed with fluorescence measurements, is less likely. Secondly, a number of important anticancer agents can be studied which have unfavourable fluorescence proper-

Table 3. Functional multidrug resistance protein (MRP) assays using daunorubicin and calcein-AM

Cell line	Daunorubicin accumulation ratio	Calcein accumulation ratio	Calcein efflux ratio	MRPr1/isotype ratio
HL60	1.05*	1.06†	1.1‡	≈ 2.5§
HL60/ADR	1.6	4.7	2.6	≈15
GLC_4	1.0	1.0	1.1	1.8
GLC_4-ADR_2	1.1	1.05	1.1	2.6
GLC_4-ADRpr	1.4	1.3	1.2	4.7
GLC_4-ADR_{150}	2.2	2.3	2.1	21.1

*Ratio of daunorubicin accumulation (2 μM, 60 min) with or without genistein (200 μM); data are from [50]. †Cells are loaded for 10 min with 0.05–0.5 calcein-AM with/without 1 mM probenecid or 100 μM benzbromarone. Data are ratios of calcein fluorescence with or without modulator (n=three experiments). ‡After loading with calcein-AM the cells were washed and calcein was expelled for 30 min with or without modulator present. Data are ratios of calcein fluorescence with/without modulator (n=three experiments). §Ratios of mean FITC fluorescence with MRPr1 primary antibody/isotype control antibody (2% formalin/acetone fixation) (n=one experiment).

ties, such as etoposide, vinca alkaloids or methotrexate. Thirdly, these assays give an absolute value of drug accumulation in (femto) moles per cell, which is difficult to obtain by flow cytometry because methods to standardise flow cytometric procedures are still evolving [22, 46, 75]. Therefore, in certain cases, for instance for verification or support of important data, radiolabel-based assays should be considered. In this way, we were able to translate a certain value for R123 efflux data in AML to a percentage increase in Dnr accumulation [46]. Also, the finding that SDZ PSC833 increased Dnr fluorescence in AML to the same extent as radiolabelled Dnr, indicated that SDZ PSC833 had no major effects on Dnr fluorescence properties at the conditions used.

If it is decided to apply a radiolabelled drug assay, experimental variables, such as viability of cells, medium composition, use of thawed cells etc., of course, have to be controlled, as in the case of fluorescence-based experiments.

Laser scanning fluorescence microscopy

An alternative approach to the detection of the uptake of fluorescent molecules in cells is the use of a fluorescence microscope. Advantages compared to flow cytometry are that a small sample number can be studied and that the intracellular distribution of fluorescent molecules can be visualised. If a laser scanning fluorescence microscope combined with computerised image processing is applied, intracellular processes can, in principle, be followed in time. The method is not suitable for high capacity screening. The number of cells that needs to be analysed will depend on the heterogeneity in the sample as to the levels of the resistance phenotypes and the percentage of resistant cells present.

In order to measure Pgp or MRP protein or function, in principle, the same molecules can be used as for flow cytometry. We have used this approach in the past to show that, in MDR tumour cell lines with Pgp or MRP overexpression, the ratio of nuclear to cytoplasmic Dox (N/C ratio) is decreased compared to the drug-sensitive parental cell lines [76–78]. This was the case even in low-resistant cells [77]. Based on these data, we undertook an analysis of AML blast cells and have found that, upon incubation of these cells with Dox, the Dox N/C ratios were significantly lower in a group of patients refractory to chemotherapy compared to responding patients [22]. A modification of this procedure might be to measure nuclear Dnr in blast cells after incubation with or without modulator, after fixation of the cells with formaldehyde, according to Willingham and colleagues [79]. With this procedure, the nuclear Dnr remains fixed, whereas the cytoplasmic Dnr is lost. Since it is the nuclear Dnr that is important for its cytotoxic action, this allows quanitification of the relevant pool. An example of such an assay is shown in Figure 2, where Pgp-negative KB3-1 cells and Pgp-positive KB8-5 cells, as well as Pgp-positive AML, are incubated with Dnr with and without SDZ PSC833 and then fixed on to cytospins. The effect of the modulator on nuclear Dnr fluorescence can clearly be seen. Although the procedure is simple and requires small tumour samples, a Dnr-based method may not be

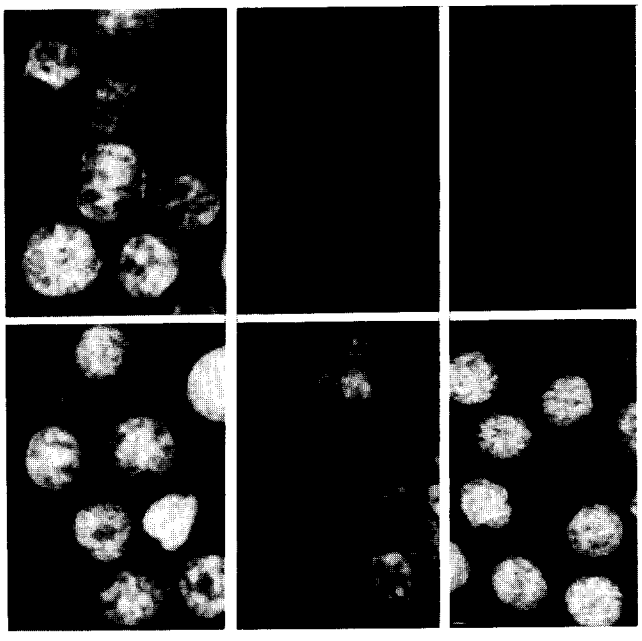

Figure 2. Fluorescence microscopic image of nuclear daunorubicin fluorescence in KB3-1 cells (left), P-glycoprotein (Pgp)-positive acute myeloid leukaemia (AML) (middle) and KB8-5 cells (right) after 75 min incubation with 2 μM daunorubicin without (top) or with (bottom) 2 μM SDZ PSC 833. Cells were cytocentrifuged and fixed for 5 min with 3.7% formaldehyde.

sensitive enough to detect the lowest (clinically relevant?) levels of pump activity. Refinement of analysis with this and other probes, nevertheless, may have potential if the goal and design of the experiments are carefully chosen. One such application might be the detection of intracellular glutathione-S-conjugates in intracellular vesicles in MRP research [80].

Pgp and MRP activity in solid tumours

Virtually no measurements of Pgp or MRP activity in patients' tumours have been done. A probe proposed for *in vivo* monitoring, which has been applied to mouse tumours, is the use of the organotechnetium complex [^{99m}Tc]SESTAMIBI, which is pumped by Pgp [81]. Experiments designed to measure *ex vivo* Pgp or MRP activity in dissociated solid tumour cells, with techniques described above for leukaemias, are now in the pioneering phase [82–83].

CONCLUSION

Recommendations for the measurement of Pgp or MRP in clinical tumour samples are, in general, to apply Good Laboratory Practice standards: to use more than one method or antibody to obtain insight into the accuracy of the results of assays or to use controls (cell lines) which undergo as many steps as possible of the same analytical procedures. Specific protocols for each method have to be proven to work in each investigator's hands. In conclusion, it is not an easy task to translate laboratory experiments into clinical practice, but if we want to learn the meaning of Pgp or any other (resistance) marker in clinical practice, then the assays have to be rigorously controlled. After many years of experience in the MDR field, we are still in this learning process. Obviously, the concept that intracellular drug concentrations might increase in Pgp-overexpressing tumour cells by co-medication with Pgp inhibitors (resistance modulators), leading to increased tumour cell kill, has not yet been proven in the clinic, especially not for the treatment of solid tumours. The reasons for this are not known, but might include the absence of Pgp in the tumour cells, which are important for responses, in the small contribution of Pgp to clinical drug resistance caused by partial (*in vivo*) inhibition of Pgp by endogenous substrates or the relative abundance of other resistance mechanisms [84–86].

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Acknowledgements—Our studies are supported by grants from the Dutch Cancer Society, the VSB grant programme and Bristol-Myers Squibb.