SEMINAR

# Diagnostics of Multidrug Resistance in Cancer

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**Multidrug resistance (MDR), caused by the overexpression of two membrane proteins, MDR1-Pgp and/or MRP, is a major obstacle in the chemotherapy of cancer. The proper laboratory diagnosis of clinical multidrug resistance is still an unresolved question, and this uncertainty, in a vicious cycle, does not allow the correct evaluation of the clinical relevance of the MDR phenomenon. Moreover, inefficient MDR diagnostics hinders the development of effective resistance-modulation strategies. In this review, after describing the basic features of the MDR drug pump proteins, the currently employed diagnostic methods are discussed. We suggest that a quantitative, functional method developed in our laboratory may provide a major help in the laboratory assessment of cancer MDR.** (Pathology Oncology Research Vol 4, No 4, 251–257, 1998)

*Key words:* P-glycoprotein, MDR1, MRP, multidrug transporters, diagnostic methods

## *Introduction*

The term "multidrug resistance" refers to a special form of clinical resistance of various tumors against a wide range of chemotherapeutic agents. This form of resistance can be attributed to an active, energy-dependent efflux of chemically unrelated cytotoxic agents, due to the activity of a group of plasma membrane transporters. Consistent with their role as drug efflux pumps, the expression of these MDR proteins (P-glycoprotein or Pgp, MDR1) and MRP1 (Multidrug Resistance Protein) confers resistance against a wide range of currently used antineoplastic drugs, by maintaining their intracellular levels below a cell-killing threshold (for reviews see 1–4)

The physiological role of the MDR proteins is probably linked to the function of an ancient-type cellular immune apparatus, providing xenobiotic resistance.[5] In connection with this role, MDR1-Pgp in mammals is expressed at high levels in the epithelial cells of the liver, kidney and most secretory organs, and was found to be present in the capillary endothelial cells of the blood-brain and blood-testis barriers.[2,4,6] MRP1[7] acts as high affinity, primary active transporter of the cysteinyl leukotriene, LTC4,[8,9] while the lack of its hepatocanalicular homologue, cMOAT (canalicular Multispesific Organic Anion Transporter, MRP2) results in a decreased transport of conjugated bilirubine as observed in the Dubin-Johnson syndrome.[10] Experiments in knock-out mice indicate that the lack of both Pgp-MDR1 and MRP is compatible with life, though an increased sensitivity to toxic agents is clearly present under these conditions.[11,12]

In most case tumors evolving from tissues with high initial MDR expression show primary resistance to chemotherapy. However, cytotoxic therapy may also be responsible for the selection and/or induction of resistant tumor cells, as secondary resistance could also be correlated with an increased level of drug pump protein expression (for reviews see 1–4, 13).

Several additional mechanisms may play a role in the development of clinical cancer drug-resistance, but the major importance of the multidrug resistance phenotype caused by the MDR proteins has become widely accepted. Assumably, the proper estimation of MDR should lead to a significantly more efficient treatment of cancerous diseases. However, the detection of the expression and function of the resistance proteins still remains a controversial issue, due to methodological differences and inconsistencies in the interpretations of laboratory results.

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### *Basic features of the MDR proteins*

The two major membrane proteins which are currently known to cause multidrug resistance are the 170 kDa P-glycoprotein (P-gp, or MDR1) and the 190 kDa multidrug resistance protein (MRP). Both proteins are members of the ABC (ATP Binding Cassette) transporter family, typically constituted of two homologous halves, each containing six transmembrane helices and a conserved nucleotide binding (ABC) domain.[1-4,14] Whereas MDR1 acts as a transporter for a wide range of hydrophobic compounds and drugs, its homologue, MDR3 predominantly transports phosphatidylcholine into the bile. In case of MRP several homologues have been characterized, but their possible involvement in drug transport has not been clarified as yet.[15]

Both MDR1 and MRP1 bind and hydrolyze ATP in connection to the transport of drugs. MDR1, localized mostly in plasma membrane, carries out only the extrusion of large, hydrophobic, uncharged or slightly positively charged molecules.[1-5] In contrast, MRP also transports negatively charged compounds, especially glutathione or glucuronate conjugates, and may sequester some of its substrates into intracellular vesicles.[16-18]

Due to their "promiscuity", the MDR proteins transport a large number of cytotoxic compounds (with different cellular targets and mechanisms of action) that are frequently applied in cancer chemotherapy. As demonstrated in *Table 1*, there is an overlapping resistance caused by MDR1 and MRP1 for several compounds, while in some cases the two MDR proteins may differ in their substrate-specificity. Therefore their selective recognition in the laboratory diagnosis is essential for any proper therapeutic intervention.

Agents which inhibit the function of MDR1-Pgp and/or MRP1, either competitively or non-competitively, may be useful in preventing clinical multidrug resistance. There is a growing interest in the use of such clinically active "MDR-modulating" or "MDR-reversing" agents, which are non-toxic but significantly increase the cytotoxic action of MDR-related drugs.[19] Several such compounds have been identified, as diverse in structure as the transported drugs, and some of them have been introduced in extensive clinical trials.[20-24] Based on these efforts, the need for a proper diagnostics of the MDR proteins has become even more apparent.

### *Methods for the detection of cancer MDR*

Several methods have been developed for the detection of the MDR phenotype, aiming for its recognition either at the mRNA level, the protein expression, or the function of the transporters.[25,26] As MDR1-Pgp and/or MRP levels in the clinical samples are generally low, and low levels of expression proved to be difficult to quantify, the sensitivity of these methods is a key issue. Moreover, these diagnostic methods have to be selective and suitable for a routine laboratory setting: they have to be simple, reproducible and quantitative in a clinically relevant fashion.

A new approach in the diagnostics of the MDR phenomenon is the use of *in vivo* radiology methods. The distribution of some Pgp-MDR1 substrate organic technetium derivatives depends on the activity of this resistance protein, thus such labeled compounds can be used as functional imaging agents in malignant bone and soft-tissue tumors,[27,28] as well as in hematologic malignancies.[29]

#### *Detection of mRNA*

The most widely used methods for the detection of MDR proteins at the RNA level are the reverse transcriptase polymerase chain reaction (RT-PCR) and the RNase protection assay.[30] Although these assays are highly sensitive, they proved to be too laborious for the routine clinical practice. An additional problem is their difficult quantitation, which complicates interlaboratory comparison and reproducibility.

#### *Detection of MDR protein expression*

A number of monoclonal antibodies have been generated against the MDR proteins.[31,32] For MDR protein detection immunoblotting and immunohisto/cytochemistry were the initial methods of choice, the reason being their relatively easy application for screening. However, these results were extremely variable due to the differences in experimental procedures and inconsistencies in interpretation. The conflicting results and methods lead to the aim of standardizing laboratory techniques. Several multicentric studies have been designed to circumvent these interlaboratory discrepancies and to elaborate consensus recommendations.[33,34]

***Table 1.* Major chemotherapeutic compounds transported by the MDR proteins***

| *MDR1-Pgp* | *MDR1-Pgp and MRP1* | *MRP1* |
|---|---|---|
| taxol | anthracyclines (e. g. doxorubicin, daunorubicin, idarubicin, mitoxantrone), vinca-alkaloids (vincristine, vinblastine), epipodophyllotoxins (e. g. etoposide and teniposide), taxanes (e. g. taxol and taxotere), actinomycin D | cisplatin and metal complexes, alkylating agents** (e. g. chlorambucil) |

* based on refs. 1–4

** the role of MRP1 in the resistance against alkylating agents is controversial, because the inactive metabolites are removed from the cells.

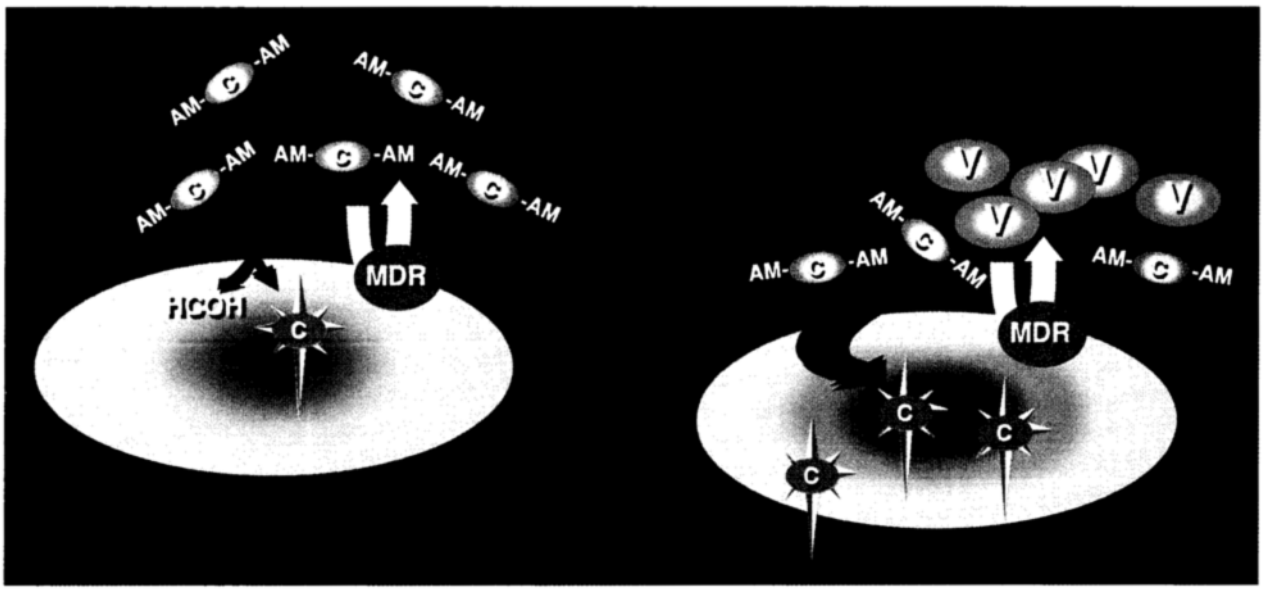



***Figure 1.*** *Model for the basic principle of the Calcein-assay. The acetoxy methylester (AM) form of Calcein (labeled as AM-C-AM in the figure) rapidly enters normal cells and after cleavage by intracellular esterases free Calcein is formed and accumulated. Cells expressing MDR proteins (MDR1-Pgp and/or MRP1) actively extrude Ca-AM, thus intracellular accumulation of free Calcein is prevented. If MDR action is inhibited e. g. by verapamil (v), Calcein accumulation occurs in MDR positive cells as well.*

Flow cytometry has also become widely used in the laboratory detection of the expression of MDR proteins. While this method can address the problem of cellular heterogeneity, its involvement in the MDR diagnostics until recently has been mostly restricted to haematological malignancies (see also 37). In this method the crucial point is the choice of the proper antibody. The best way for the evaluation of the expression level of the MDR cell surface proteins is the use of monoclonal antibodies which bind to extracellular epitopes, where permeabilisation of the cells is not required. While several monoclonal antibodies which recognize extracellular epitopes of Pgp-MDR1 are already available, until to date no useful antibody against an extracellular epitope of MRP has been reported. Also, some of the anti-Pgp-MDR1 antibodies (especially UIC2) were shown to be sensitive to the actual conformation of the protein, which further complicates their clinical application.[38]

A large multicentric study, including 36 French haematological centers[39] has drawn the attention to the importance of antibody concentration, and interpretation in the MDR determinations. One of the general conclusions emerging from these efforts is the emphasis on multiparameter analysis, and especially the requirement of using a dual (i.e. functional and morphological) detection approach.

*Functional MDR assays*

Functional assays share the advantage that they measure transport activity, a clinically relevant property of the drug transporter MDR proteins. The most widely used functional assays are based on the radioactively labeled or fluorescent drug/dye extrusion measurements. These include estimation of labeled vincristine or ADR uptake, as well as the change of cellular fluorescence during accumulation of fluorescent MDR1 substrates, e.g. anthracyclines, verapamil- derivatives, rhodamine 123, some Hoechst dyes, or Fluo-3 (for reviews see 33-35,40). Flow cytometry may provide outstanding possibilities in the functional determination of the MDR phenotype.

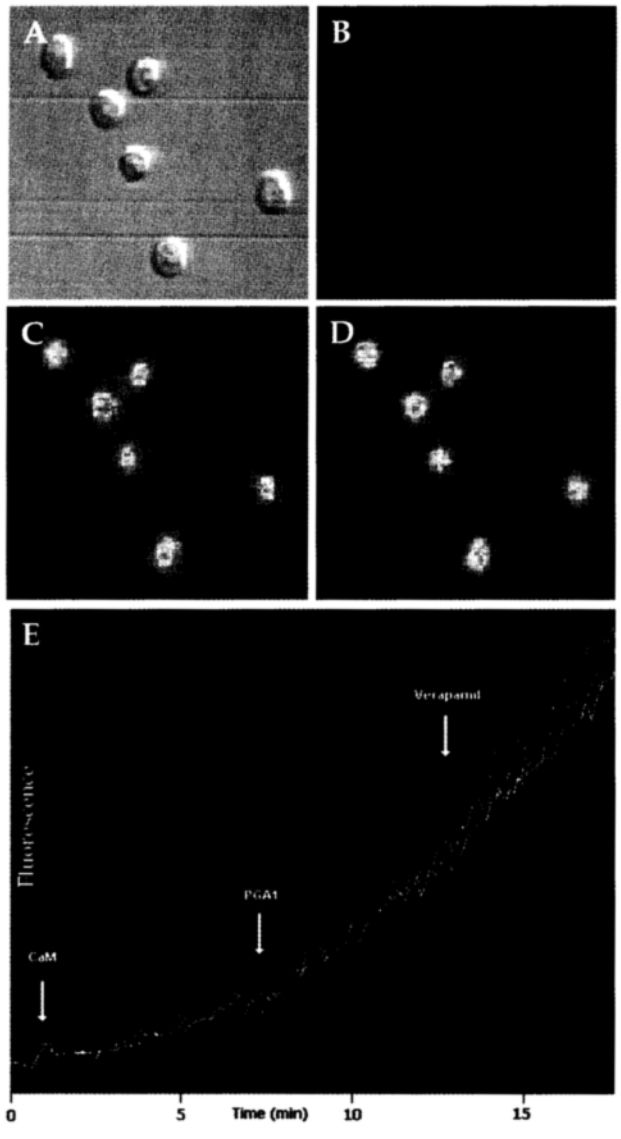
The use of fluorescent dyes in measuring MDR function is hindered by the facts that the fluorescence of most of the above compounds depends on cellular pH and intracellular free ions, and their cellular distribution is altered and fluorescence is quenched upon binding to DNA or other cell components. The widely used rhodamine 123 efflux assay involves a preloading of the cells with this fluorescent dye and then measuring its drug-pump dependent extrusion.[35,41,42] Although the sensitivity of this method is higher than that of the fluorescent anthracycline measurements, the proper quantification of the transport activity is hindered by a non-MDR1-mediated efflux and by non-specific changes in rhodamine 123 fluorescence.

***A quantitative functional MDR-diagnostic method: the Calcein assay***

It has been demonstrated in our laboratory that MDR1 expressing cells show a decreased uptake of the fluorescent cell viability dye, Calcein, as the hydrophobic Calcein-AM is actively extruded by the multidrug transporters

before its intracellular conversion to free Calcein.[43,44] Free Calcein is a poor substrate of the multidrug transporters thus it is well retained in the cells. When the extrusion is blocked by an inhibitor (e.g. verapamil), Calcein AM becomes trapped intracellularly upon conversion to free Calcein by nonspecific cytoplasmic esterases, and fluorescent free Calcein rapidly accumulates, just as in the control cells *(Figure 1)*.

This method can be efficiently used in flow cytometry, and the observed difference in the rate of accumulation of cellular fluorescence is characteristic for the MDR protein drug transport activity. Calcein does not bind to cellular



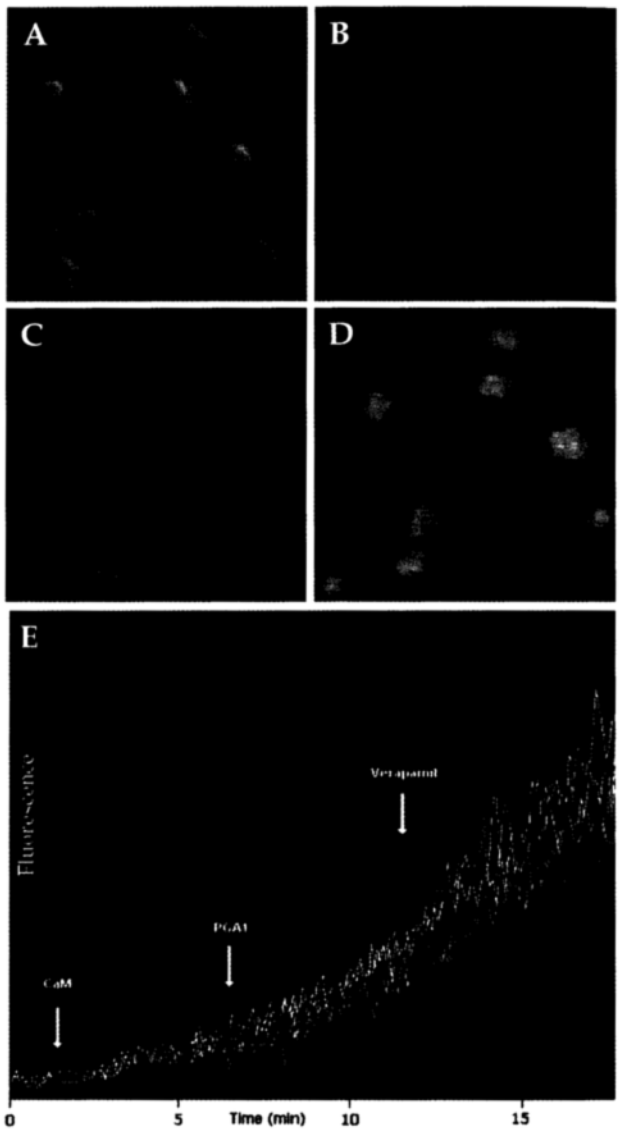




***Figure 2. and Figure 3.*** *Diagnostics of MDR by single cell fluorescence imaging. Bone marrow cells of a patient (Panel A, DIC image) were subjected to Calcein AM, and fluorescence images (Panels B-D) were recorded in every 5 seconds. Panel B: 5 min after the addition of CaAM; panel C: 5 min after the addition of Prostaglandin-A1 (PGA1), an MRP inhibitor; panel D: 4 min after the addition of verapamil, a non-selective MDR inhibitor. In panel E the records for the fluorescence values and the addition of compounds (indicated by arrows) are demonstrated. Each plot represents the sum of pixel values (in arbitrary units of fluorescence) in the cells selected on the DIC image. For details see ref. 48.*

***Figure 2.*** *Bone marrow cells of a patient with drug-resistant leukemia ( AML). After the addition of PGA1 a maximum increase in the rate of fluorescence development is observed – these drug-resistant cells express mostly MRP1.*

***Figure 3.*** *Bone marrow cells of a patient with drug-resistant leukemia (ALL). After the addition of PGA1 some increase in the rate of fluorescence development is observed, which is further increased by verapamil – these drug-resistant cells express both MRP1 and MDR1-Pgp.*

components but has a bright fluorescence, practically insensitive to changes in pH, as well as $Ca^{2+}$ or $Mg^{2+}$, which eliminates some of the drawbacks of other fluorescence dye assays.[45] We have also demonstrated that a quantitative measure of transport activity, the MDR activity factor, MAF, can be calculated from the rate of Calcein transport in the presence and absence of an inhibitor (e.g. verapamil) and given as a dimensionless value.[45]

As both Calcein AM and free Calcein are transported by MRP,[46,47] inhibitors of MRP can also be examined by this method. Moreover, a parallel and quantitative estimation of the expression and function of these drug transporters can be performed by using a selective MRP inhibitor in this assay.[48] The MDR pump Calcein assay in flow cytometry has been applied by now in several research and clinical laboratories.[4,25,40,42,49,50]

In *Figure 2* and *3* we demonstrate the use of Calcein assay in single cell fluorescence studies, by examining the presence of the MDR pump proteins in the bone marrow samples of leukemic patients. As demonstrated in *Figure 2*, the tumor cells in this case expressed clinically relevant amounts of the MRP1 protein, as reflected by the PGA1-sensitivity of the Calcein accumulation. In contrast, the tumor cells examined in the experiment shown in *Figure 3*, expressed both MRP1 and MDR1-Pgp (for details see the Figure legend).

As a summary, a selective, quantitative diagnostics of both MDR1 and MRP can be performed by using the Calcein assay, either in flow cytometry or single cell measurements. Such a laboratory assay should be a major help in deciding the appropriate drug combinations to be used in the treatment of a given tumor.

### *Clinical significance of a proper MDR diagnostics*

A large number of studies have examined the correlation between Pgp-MDR1 expression and function, and the treatment failure in haematological malignancies. Despite numerous attempts, the interpretation of data from different centers has been difficult to harmonize. Multivariate analyses have demonstrated significant correlation between CD34 positivity and Pgp-MDR1 expression.[50,51] The level of expression as well as the functional activity of MDR1 at diagnosis was shown to have a prognostic impact in de novo, refractory or relapsed acute myeloid leukemia (AML),[36,50-57] while the results concerning Pgp-MDR1 expression in acute lymphoblastic leukemia (ALL) are more controversial.[58] Still, the role of Pgp-MDR1 as a prognostic factor in achieving remission and longer survival in several haematological conditions has been established. The clinical relevance of MRP1 expression is less clear,[59] though a subtype of AML which is associated with the inversion of chromosome 16 (disrupting the MRP1-gene) has a favorable prognosis and is more chemosensitive.[60]

The ultimate goal of assessing the MDR phenotype is that these transporters may be possible targets of future therapeutic efforts. Early trials for drug-resistance modulation were initiated in the 1980s, right after the discovery of the chemosensitizer action of verapamil and other related agents which were already used in clinical practice. The aim of these clinical trials was to achieve better outcome (i.e. higher complete remission rates and longer survival) by combining intensive chemotherapy with the chemosenzitizers in leukemic patients, without increasing toxicity and mortality.

Both verapamil and cyclosporin A were applied in various tumor patient groups treated with combination chemotherapy.[20-23,61,62] Encouraging results were recorded concerning the use of PSC-833, a non-immunosuppressive cyclosporin analogue combined with Mitoxantron- and Vepesid-containing salvage regimen in relapsed or refractory AML patients.[63] In a recently published randomized trial, quinine, another Pgp-MDR1 modulator, was found to increase the complete remission rate and survival in Pgp-MDR1 positive cases of high risk myelodysplasia (MDS) patients, treated with intensive chemotherapy.[64] The fact that quinine had this effect only on Pgp positive MDS, suggests a specific, Pg-MDR1 mediated drug resistance mechanism, and underlines the importance of a proper diagnosis. Combined with an improved preliminary diagnostic evaluation of the multidrug resistance phenotype, new agents may soon become effective tools in the fight against drug-resistant cancer.

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