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ORIGINAL ARTICLE

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Multidrug resistance: molecular mechanisms and clinical relevance

Abstract Multidrug resistance (MDR) describes the phenomenon of simultaneous resistance to unrelated drugs. It has been a decade since the P-glycoprotein (Pgp) gene, which is associated with a form of MDR caused by reduced drug accumulation, was cloned. Thus, this would seem to be an appropriate time to evaluate our understanding of this form of MDR. The two MDR genes identified in humans to date (the MDR-associated protein [MRP] and Pgp genes) are structurally similar and both are members of the ATP-binding cassette (ABC) transporter family. Although the physiological role of MRP is not yet understood, one Pgp gene (*mdr1*) plays an important role in the blood-tissue barrier and the other (*mdr2/3*) is involved in phospholipid transport in the liver. A variety of compounds (chemosensitizing agents) can interfere with Pgp and MRP function; such agents may improve the efficacy of conventional therapy when used in combination with such regimens. Determining the roles cellular MDR mechanisms play in patients' response to chemotherapy is a major challenge. Using Pgp and MRP as molecular markers to detect MDR tumor cells is technically demanding, and solid tumors in particular contain heterogeneous cell populations. Since MDR requires Pgp or MRP gene expression, clinically relevant gene expression thresholds need to be established; sequential samples from individual patients are valuable for correlating MDR gene expression with the clinical course of disease. Studies in leukemias, myelomas, and some childhood cancers show that Pgp expression correlates with poor response to chemotherapy. However, in some cases, inclusion of a reversing or chemosensitizing agent such as verapamil or cyclosporin A has improved clinical efficacy. Such agents may inactivate Pgp in tumor cells or affect Pgp function in normal cells, resulting in altered pharmacokinetics. It would be interesting to determine whether patients who fail treatment in the presence of chemosensitizing agents acquire other MDR mechanisms. The ABC transporter superfamily in prokaryotes and eukaryotes is involved in the transport of substrates ranging from ions to large proteins. Of the 15 or more ABC transporter genes characterized in human cells, two (Pgp and MRP) cause MDR. Therefore, it would be relevant to determine the number of such genes present in the human genome; however, extrapolating from the number of ABC transporter genes in bacteria, the human gene probably contains a minimum of 200 ABC transporter superfamily members. Thus, tumor cells can potentially use many ABC transporters to mount resistance to known and future therapeutic agents. The challenge will be to determine which ABC transporters are clinically relevant. Despite the potential of tumor cells to protect themselves, a variety of malignancies can be successfully treated with chemotherapy. This may provide unique insights.

Key words Multidrug resistance (MDR) · P-glycoprotein · ABC transporters · Anticancer drugs

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Introduction

Multidrug resistance (MDR) describes the phenomenon of simultaneous resistance to unrelated drugs. Extensive studies with cell lines and transplantable tumors have shown that MDR can develop rapidly. Genes involved in MDR have been identified in well-characterized experimental systems, and their role in drug resistance has been confirmed by gene transfer. Mechanisms identified to date include reduced drug accumulation, involving the P-glycoprotein (Pgp; *mdr1* gene) [10] and the MDR-associated protein (*mrp*) gene [11]; drug detoxification, involving the glutathione-S-transferase gene [22]; altered targets, involv-

ing topoisomerase II [1]; and alteration in drug-induced apoptosis, involving genes in the Bcl-2 pathway [24]. A variety of other molecular markers have also been shown to be associated with MDR cells, although the roles they play are not understood. In some instances where MDR develops as a result of gene amplification, overexpression of the flanking genes located in the same amplicon as the causative gene can occur. Such genes often add to the complexity and heterogeneity of MDR cells, but they do not contribute significantly to the drug-resistance phenotype.

Cancer chemotherapy has its roots in antimicrobial chemotherapy, and many concepts are applicable to both. Paul Ehrlich, the father of chemotherapy, lamented that drug resistance followed the development of new drugs "like a faithful shadow" after years of experiments developing antimicrobial drugs. As we forge ahead with the development and application of more effective anticancer drugs, it is likely that drug-resistance mechanisms (mechanisms that are routinely used by normal cells for defense against xenobiotics) will be enlisted by tumor cells for their own protection. Therefore, it is relevant to evaluate whether the MDR mechanisms identified in experimental systems play a significant role in the response of patients to cancer chemotherapy.

The question as to what role cellular MDR mechanisms play in patients' responses to cancer chemotherapy is not a simple one because factors such as tumor burden, tumor heterogeneity, variation in drug detoxification, and drug clearance may affect response in addition to the drug sensitivity of the tumor cells. In general, the cellular MDR mechanisms characterized to date play a rate-limiting role in response to drugs in experimental systems such as transplantable tumors or cells in culture. However, whether they play a similar role in the clinical response is only beginning to be explored in a number of malignancies. The focus of this presentation is MDR associated with Pgp, with some reference to MRP. The *mdr1* and *MRP* genes are members of a superfamily of ATP-binding membrane-associated transport protein [ATP-binding cassette (ABC) transporters] genes widely distributed in all kingdoms of life [10].

Pgp structure and function

Pgp and MRP are the two members of the mammalian ABC transporter protein family identified to date that confer MDR [10]. Both Pgp and MRP are large membrane proteins containing more than 1200 amino acids, and both are predicted to contain multispanning transmembrane segments and two highly conserved ATP-binding domains [10]. The predicted topology of Pgp indicates that the N- and C-terminal halves are similar, each containing six predicted transmembrane segments and an ATP-binding domain. The predicted structure of MRP is different, with eight predicted transmembrane segments being in the N-terminal half and four in the C-terminal half [11]. The genes encoding MRP and Pgp are evolutionarily very distant. The *MRP* gene is more closely related to the cystic fibrosis gene, CFTR, whereas the *mdr1* gene is evolutionarily more related to the bacterial hemolysin B gene than to the mammalian *MRP* gene. Thus, it is not possible to predict substrate specificity or whether a particular ABC transporter is capable of producing an MDR phenotype from structural information alone.

The mechanism of action of Pgp has been investigated extensively from a biochemical perspective in the past few years [31]. Due to the broad spectrum of drugs to which MDR cells overexpressing Pgp are resistant, it has been speculated that Pgp acts via indirect mechanisms such as altering intracellular pH or modifying membrane potential. The use of purified Pgp and functionally reconstituting it into liposomes to investigate its properties show that Pgp alone is sufficient to transport different drugs. It was possible to demonstrate that energy for transport is derived from magnesium ATP and an intrinsic Pgp ATPase. Using membrane vesicles containing high levels of Pgp or purified Pgp reconstituted into liposomes, it has also been possible to demonstrate that Pgp recognizes and removes the fluorescent substrate Hoescht 33342 from the lipid phase of the membrane bilayer [31]. Furthermore, the use of a protonophore and measuring the intraliposomal pH during transport showed that a pH gradient is not required for drug transport.

These findings are consistent with a model of Pgp action in which Pgp interacts directly with a broad range of hydrophobic compounds in the lipid bilayer and transports them directly into the exterior of the cell using ATP and the intrinsic ATPase for energy. Such a model is consistent with genetic studies of Pgp showing that point mutations located within some predicted transmembrane segments affect drug specificity and response to reversing agents. The concept that the transmembrane domain of ABC transporters dictates substrate specificity has been corroborated in mutation studies in the bacterial hemolysin transport system, in which point mutations in the transmembrane domain of hemolysin B significantly alter substrate specificity [32].

Efforts to purify and reconstitute the MRP protein are currently ongoing. It would be very interesting to determine whether MRP can recognize drugs directly, similar to Pgp, or whether additional modification, such as conjugation of the drug, is required. A number of studies have implicated MRP as a "GS-X pump," suggesting that it recognizes glutathione-conjugated compounds [16–18]. Other studies suggest that MRP recognizes and transports compounds such as daunorubicin directly. All these studies were undertaken with whole cells or membrane vesicles. A purified system may allow the core function of MRP to be delineated.

Pgp is encoded by two genes in humans (*mdr1* and *mdr2/3*) and by three in rodents [10]. These genes are closely linked on chromosome 7, and the selection of cell lines for high levels of MDR can result in the amplification of the *mdr* genes and some flanking genes. The *mdr1* gene causes drug resistance, whereas the *mdr2/3* gene does not appear to do so. The elegant studies of Borst and Schinkel [3] using homologous recombination inactivation in knock-

Table 1 Techniques used for detection of Pgp or *mdr1* in clinical samples[a]

Northern blot
RNase protection
RNA slot-blot
Reverse transcriptase-polymerase chain reaction
RNA in situ hybridization
Immunoblot
Immunohistochemistry
Immunofluorescence staining

[a] Clinically relevant level of expression needs to be established

out mice have provided significant insights into the function of the Pgp-encoding gene families in rodents [3]. Knockout of the equivalent of the human *mdr1* gene in mice has demonstrated that this gene is involved in the blood-brain barrier and in the transport of drugs in the intestinal tract [27, 28]. Knockout of the analogue of the *mdr2/3* gene in mice has shown that this gene is involved in the transport of phosphatidylcholine in the bile canaliculi of liver [33]. These studies demonstrate that the different Pgp isoforms have different functions. Moreover, these studies in knockout mice have demonstrated that functional Pgp-encoding genes are not essential for development but appear to be important for the normal function of the adult animal.

Correlation of Pgp and response in human cancer

Transfection of the *mdr1* gene results in MDR, indicating that the gene product, Pgp, may be used confidently as a molecular marker for the presence of MDR cells in patients' biopsies. To determine whether the level of Pgp expressed correlates with response to chemotherapy, a method that reliably detects the presence of Pgp in biopsy samples needs to be employed. In general, the level of mRNA expressed correlates with the level of protein, although exceptions have been reported.

Table 1 lists some of the approaches used for the detection of Pgp in clinical samples. In evaluations of the utility of these techniques a number of considerations are relevant: (1) because the MDR phenotype is dependent on the level of gene product expressed rather than on specific mutations, detection must be at least semiquantitative; (2) it must be highly sensitive and specific; (3) it must have the ability to detect Pgp at the single cell level such that the level of expression in normal cells and tumor cells can be differentiated; and (4) it should ideally be applicable to formalin-fixed paraffin-embedded material, as this would allow the detection and evaluation of Pgp expressed in material routinely processed through a pathology department. Moreover, a method incorporating the latter would allow the evaluation of archival material for retrospective studies.

In addition to measuring the level of Pgp expression, in malignancies such as leukemias it is possible to use flow cytometry to determine whether an altered drug accumulation mechanism can also be detected in parallel with Pgp expression. In this manner, independent lines of evidence can be used to confirm the presence of the MDR phenotype in tumor cells.

Numerous studies have been published on different approaches to detect MDR tumor cells in clinical samples (for reviews see [4, 21]). The conclusions from these studies have not always agreed with respect to the proportion of patients considered to have a significant level of *mdr1* tumor cells. This is due in part to the lack of a universal standard for quantifying expression. Moreover, it is difficult to compare one study with another due to the different methodologies used [2]. However, a pattern has emerged from individual studies. If Pgp is detected in a tumor at diagnosis, the level of expression is usually higher in subsequent relapses. On occasion, Pgp is not detected at presentation but upon subsequent relapses. Thus, the opportunity to obtain sequential samples from a single patient during the course of the disease is valuable when one attempts to correlate the expression of any MDR-associated gene with the clinical course of the disease. Regardless of which technique is used for the molecular detection of an MDR mechanism, thresholds for clinically significant levels of expression must be established by the use of appropriate clinical designs and studies.

A number of studies have convincingly supported the concept that the presence of Pgp in tumor cells correlates with poor prognosis for therapy. The most supportive observations have been made in leukemias and in pediatric cancers. For example, using an anti-Pgp monoclonal antibody, Ma et al. [20] were the first to demonstrate that Pgp may be overexpressed in acute myelogenous leukemia (AML). In two patients, leukemia cells were negative for Pgp at diagnosis of AML, but one patient became positive at first relapse and the other on recovering from second induction chemotherapy. Subsequently, two large studies of 61 and 63 patients, respectively, correlated *mdr1* mRNA expression with response to treatment in acute leukemia [23, 25].

In pediatric cancers the work of Chan and colleagues [6–8] has documented a significant correlation between Pgp detection using immunocytochemistry and poor prognosis and, significantly, lack of Pgp detection and good prognosis in pediatric sarcoma and neuroblastoma. Recently, using an RNA slot-blot assay, Savaraj et al. [26] showed that *mdr1* expression at diagnosis in small-cell lung cancer (SCLC) correlates with poorer outcome (median survival of 2 months) as compared to *mdr1*-negative patients (median survival 10 months). The correlation of *mdr1* expression with poor outcome in SCLC is a surprise. It has generally been assumed that *mdr1* is not involved in this cancer since cell lines established from SCLC cells express little *mdr1*. MRP is generally thought to be involved in lung cancer, and further investigation of the *mdr1*-negative patients may identify patients in that category. However, it should be noted that many investigators have not found such correlations and that such negative findings may not always be reported. The reasons for this are not clear, although some

Table 2 Pgp (*mdr1*) expression and clinical outcome

1) No significant correlation with outcome
· Assay and clinical trial design technically sound?
· Other MDR mechanisms involved?
2) Correlation with outcome: patients with Pgp-positive tumors have poorer outcome than patients with Pgp-negative tumors, but few long-term survivors overall
· Other mechanisms of MDR involved?
3) Correlation with outcome: patients with Pgp-positive tumors associated with poor outcome and those with Pgp-negative tumors associated with long-term survival
· Good candidate for reversal of Pgp trial?
· Other MDR mechanism present in Pgp-positive tumors?

negative findings may be due to the difficulties involved in establishing an appropriate assay for detection of MDR [2]. Therefore, it is important that investigations are undertaken in which the detection assay, preparation of the clinical material, and clinical design of the study are rigorously controlled.

The different categories of outcome and their implications are shown in Table 2. Different malignancies may yield different results. Moreover, the particular MDR mechanism investigated may not necessarily be the one that is limiting response in the patient; in such instances, no clinically significant correlation should be observed. In this context it is now increasingly evident that more than one MDR phenotype may be expressed in an MDR cell [29, 36]. In earlier studies, selection for highly drug-resistant cell lines resulted in gene amplification, and a single MDR mechanism (e.g., Pgp overexpression) usually predominated. The lower level of expression of other MDR mechanisms is often masked. Such a situation is not the norm in clinical samples, in which it is possible that even low expression of Pgp or other MDR mechanisms may be significant. Such considerations further emphasize that the clinical significance of any MDR mechanism expressed in clinical samples needs to be validated by clinical studies.

MDR reversal

A large number of compounds can reverse the Pgp-mediated MDR phenotype [14]. The initial discovery by Tsuruo and colleagues [35] that verapamil (VP) could reverse Pgp-mediated MDR provided a practical reagent for testing directly the hypothesis that the presence of such MDR tumor cells limits successful chemotherapy. Application of Pgp-reversing agents in combination with conventional chemotherapy has met with limited success. In some instances, increased toxicity has been observed, suggesting that the inclusion of a Pgp-reversing agent has affected the pharmacokinetics of the chemotherapeutic agent, probably as a result of inhibition of Pgp in normal tissues. Nevertheless, some encouraging results have been reported.

Two studies in multiple myeloma [13, 34] have suggested that the use of VP or cyclosporin A (CSA) as reversing agents in Pgp-positive patients appears to induce a response to the same drug combination (VAD) to which the disease was previously unresponsive. Pgp-negative patients were not affected by the inclusion of the reversing agents. In another study the use of CSA in combination with a modified regimen of arabinose-C and daunorubicin in AML patients resulted in a complete remission rate of 62% and a median remission duration of 13 months [19]. Such studies strongly support the hypothesis that the presence of Pgp-positive tumor cells plays an important role in limiting response in these malignancies. They also raise further important questions, e.g., what is the basis of nonresponse for those patients who are Pgp negative? In addition, it would be important to determine whether patients who fail in the presence of CSA or VP acquire other MDR mechanisms.

In pediatric cancers a recent study in retinoblastoma with a small group of patients has demonstrated that the inclusion of CSA in chemotherapy significantly increases long-term survival rates [9]. In this study it was not possible to determine the Pgp status of the tumor cells because obtaining biopsies was not an option. However, in other studies involving enucleation of the affected eye, significant Pgp expression was routinely observed. Cell lines established from such tumors also expressed Pgp [5].

How CSA functions in improving chemotherapeutic efficacy in retinoblastoma patients is not clearly understood. It is possible that CSA may inactivate Pgp in tumor cells, rendering them drug-sensitive. Alternatively, CSA may affect Pgp function in normal cells, resulting in altered pharmacokinetics and, possibly, opening up pharmacologic sanctuaries. Further investigations will be required to differentiate between these possibilities.

These studies using Pgp-reversing agents represent only the early stages in our understanding of how to use such compounds effectively. Taken together, the results to date are encouraging. They have also provided a number of guiding principles for future studies. First, in any clinical study involving the use of MDR-reversing agents the MDR status of the tumor cells should be determined on the basis of previously determined clinically relevant thresholds of expression. MDR-positive patients can then be compared with MDR-negative patients for response. Long-term follow-up should be undertaken and biopsies obtained at relapse to determine whether the original MDR mechanism is expressed. If it is not, one can assume that the reversing agent has been successful in helping to eradicate tumor cells containing the original MDR mechanism but that another mechanism may now have come into play. The pharmacokinetics of the reversing agent and the anticancer drugs should be determined to elucidate how inclusion of the reversing agents has affected drug clearance, delivery, and distribution.

ABC transporters and future prospects

It is now recognized that the Pgp- and MRP-encoding genes belong to a superfamily of genes that encode ATP-binding

membrane transport proteins in diverse species [10]. This so-called ABC transporter family contains more than 100 identified members, with representatives from the prokaryote and eukaryotic kingdoms. In eukaryotes a wide substrate specificity has been found, with different ABC transporter proteins being involved in the translocation of ions, metals, amino acids, peptides, steroid hormones, phospholipids, and drugs. Many of the ABC transporters that have been characterized were discovered during the course of unrelated genetic or biochemical investigations into medical disorders. Their postulated function has been linked to the nature of the disorder and their localization in different organs and tissues. In other cases, ABC transporters have been discovered by accident or by homology cloning. Although it is possible to determine the expression of these genes in different cell types and tissues, their functions are generally not known. However, it is clear that the superfamily of ABC transporters in prokaryotes and eukaryotes involved in the transport of a wide diversity of substrates.

It would be relevant to determine the number of ABC transporters in the human genome. Extrapolating from the known number of ABC transporter genes in bacteria, one may predict that the human genome contains a minimum of several hundred ABC superfamily members. If this is the case, tumor cells have the potential to call upon a large number of ABC transporters to mount resistance to known or future therapeutic agents. It will be challenging to determine which ABC transporters are clinically relevant.

Historically, drug-resistance mechanisms have been identified by investigating cell lines or model systems selected for resistance to specific drugs. Whether such model systems result in the identification of most or all resistance mechanisms expressed in tumor cells remains to be determined. One may speculate that due to their known genomic instability and high degree of heterogeneity, tumor cells may express drug-resistance mechanisms not observed in experimental systems. As one test of this hypothesis, we have examined blast cells from AML patients for their heterogeneity in drug transport, using a functional assay of daunorubicin uptake into these cells and their inhibition by Pgp-reversing agents or other compounds [15].

We compared CSA, a known Pgp inhibitor, and potassium cyanide (KCN), an inhibitor of oxidative phosphorylation, for their ability to increase daunorubicin uptake into the AML blast cells. KCN depletes cellular ATP and inhibits all ABC transporters, including other energy-dependent processes. This approach was originally used to demonstrate the ATP dependence of Pgp [30] and has more recently been shown to inhibit drug efflux via MRP [12]. Such generalized ATP depletion over a short period does not affect cell viability, and intracellular ATP can easily be restored in the presence of KCN by the addition of metabolizable sugars such as glucose. The modulating effects of CSA and KCN on daunorubicin accumulation in a number of cell lines and in AML patient blast cells were compared. In some cases, neither agent increased drug accumulation, indicating that energy-dependent processes were not involved. In other cases the CSA and KCN effects were roughly comparable, suggesting the involvement of Pgp only or of a closely related transporter.

In striking contrast to the cell lines, many patient samples showed very little modulation of daunorubicin uptake by CSA but large increases with KCN. We speculate that when KCN is used as a modulating agent the daunorubicin accumulation assay sums the activities of all energy-dependent processes that reduce drug uptake, whether the be ABC transporters or other mechanisms. If this interpretation is correct, then some AML patient samples show daunorubicin efflux rates equivalent to those seen in a highly vinblastine-resistant CEM cell line, and we hypothesize that some novel ABC transporters may be involved. In support of this possibility, we have been capable of demonstrating that at least one novel ABC transporter recently identified in our laboratory is expressed at quite variable levels in AML blast cells (Zhang et al., unpublished observations). Determination of whether expression of such an ABC transporter might correlate with clinical response in AML patients will require further investigation.

Despite the tremendous potential of tumor cells to become resistant to current anticancer drugs and anticancer drugs of the future, we are encouraged by the observation that some cancers are curable by chemotherapy. Understanding the basis of this may provide significant insights. It is possible that the repertoire of resistance mechanisms that a cancer cell can enlist is not limitless. By systematically checking each cancer for the possible drug-resistance mechanisms involved, we may be capable of making rational recommendations to improve the efficacy of chemotherapy against such resistant tumors using appropriate reversing agents. We are optimistic that in the future we will eventually outdistance Ehrlich's "faithful shadow" of drug resistance.

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