Anti-Cancer Drugs 1997, **8**, pp. 17–25

Review paper

The role of multidrug resistance-associated protein (MRP) expression in multidrug resistance

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Multidrug resistance (MDR) is a major hindrance to the successful treatment of neoplastic disease. The development of resistance to multiple chemotherapeutic drugs is a complex phenomenon which has been described in both tumor cell lines and human cancers. To date, two mechanisms associated with overexpression of membrane glycoproteins that function as energy-dependent efflux pumps to reduce intracellular drug levels have been identified for MDR. The first described was the product of the *MDR*1 gene, P-glycoprotein. The second mechanism is mediated by overexpression of the multidrug resistance-associated protein (MRP). While these proteins both belong to the ATP-binding cassette superfamily of transporters, they are only distantly related. Despite this low homology, they mediate resistance to a similar range of chemotherapeutic drugs. While P-glycoprotein has been well described in the literature, much less is known about the recently identified MRP. This review gives an overview of the characteristics of MRP at both the phenotypic and genotypic levels, and discusses its possible relevance in drug-refractory cancer.

Key words: Multidrug resistance, multidrug resistance-associated protein.

Introduction

Chemotherapy for the treatment of human cancers has vastly improved patient survival over the last 40 years. Whilst this treatment has been particularly successful for certain types of tumors, there is still a significant proportion that do not respond, or relapse, following chemotherapy. Multidrug resistance (MDR), whereby tumor cells display simultaneous resistance to a range of hydrophobic natural product drugs, has been described in both drug-selected cell lines and human tumor samples.[1] *In vitro* studies have shown that MDR cells differ from their drug-sensitive counterparts by (i) reduced accumulation of drug, (ii) changes in the expression and/or activity of specific cellular proteins, and (iii) intracellular alterations, such as a change in pH.[2] One of the first mechanisms of MDR described was mediated by the product of the *MDR*1 gene, P-glycoprotein.[1] P-glycoprotein is a 170 kDa integral membrane protein which has been shown to function as an ATP-dependent drug efflux pump.[3] While increased levels of P-glycoprotein were identified in many laboratory-derived drug-resistant cells[4] and some human tumors,[5,6] it became evident that other mechanisms were also responsible for this phenotype in human cancer cells.[7–13] The multidrug resistance-associated protein (MRP) is a relatively new member of the family of ATP-binding cassette (ABC) membrane transporters, and, similar to P-glycoprotein, mediates resistance to a range of structurally and functionally unrelated agents.[1,14,15]

MRP characterization

Structure and function

The *MRP* gene was cloned from a non-P-glycoprotein-mediated multidrug-resistant small cell lung cancer cell line, H69AR, selected for resistance to doxorubicin.[11,14] Using a differential hybridization approach, a 6.5 kb mRNA species overexpressed in H69AR cells as compared to the parental cell line was identified. The mRNA species was cloned and sequenced, and found to encode for a 190 kDa membrane protein with a predicted sequence of 1531 amino acids.[14,16] The *MRP* gene has been

This review was undertaken in the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York, USA, during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer.

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localized to chromosome 16p13.1.[14,17,18] Like P-glycoprotein, MRP is a member of the ABC protein superfamily. However, MRP and P-glycoprotein share only 15% amino acid identity, even though they confer resistance to a similar, but not identical, range of chemotherapeutic drugs.[15,19,20] A number of independent reports have established a functional role for MRP, by demonstrating that increased expression of the *MRP* gene correlated with increased expression of both MRP protein and resistance to MDR-associated drugs in cell lines transfected with this gene.[15,21,22]

The predicted membrane topology of the MRP protein is not conclusive. Based on the original sequence data, MRP was predicted to contain eight transmembrane domains in the N-terminal half of the molecule and four in the C-terminal half, with both portions of the molecule predicted to be *N*-glycosylated.[14,16] Due to the high sequence homology and hydropathy profile alignment of the recently identified sulfonylurea receptor (SUR)[23] and the yeast cadmium resistance protein (YCF1)[24] to MRP, this model has now been modified to contain 11 or 12 transmembrane segments in the N-proximal half and six transmembrane segments in the C-proximal half (Figure 1).[25,26] In contrast, another model has recently been proposed for the membrane topology of MRP. Bakos *et al.*[27] have suggested that human MRP contains a tandem repeat of six transmembrane helices, not dissimilar to the cystic fibrosis transmembrane conductance regulator (CFTR), each containing a nucleotide binding domain with a glycosylated C-terminal membrane bound region. In addition, a membrane-bound, glycosylated region with four to five transmembrane helices was also predicted in the N-region of MRP, which would give a total of 10 or 11 transmembrane segments in the N-proximal half of the molecule and six in the C-proximal half. This appears to be a common feature of other ABC transporters that are close relatives of MRP, such as YCF1. Accurate determination of membrane topology of proteins, such as MRP and the other ABC transporters, has not been possible due to the difficulty of crystallizing these large molecules.

Human MRP is an *N*-linked glycosylated protein which contains 12 potential glycosylation sites. Almquist *et al.*[28] demonstrated that cellular drug levels were not altered in cells treated with a glycosylation inhibitor. The functional significance of these glycosylation sites is not yet known. One possibility is that glycosylation is involved in the processing and stability of *MRP* mRNA. In addition to glycosylation, a number of groups have demonstrated that MRP is phosphorylated *in vivo*.[28,29] A functional role for protein phosphorylation of other ABC transporters, such as the cystic fibrosis TM conductance regulator, has led to suggestions that this may also be important in drug transport.[30] It has not been established, however, which amino acids in MRP are phosphorylated nor which kinases are responsible, although MRP does contain a number of sequence motifs which suggests that phosphorylation of this protein may be regulated by several kinases.[14]

To date, limited information is available about regulation of the *MRP* gene. Amplification of this gene has been identified in a number of laboratory-derived drug-resistant cell lines that display overexpression of MRP.[14,17,31–33] Despite the fact that

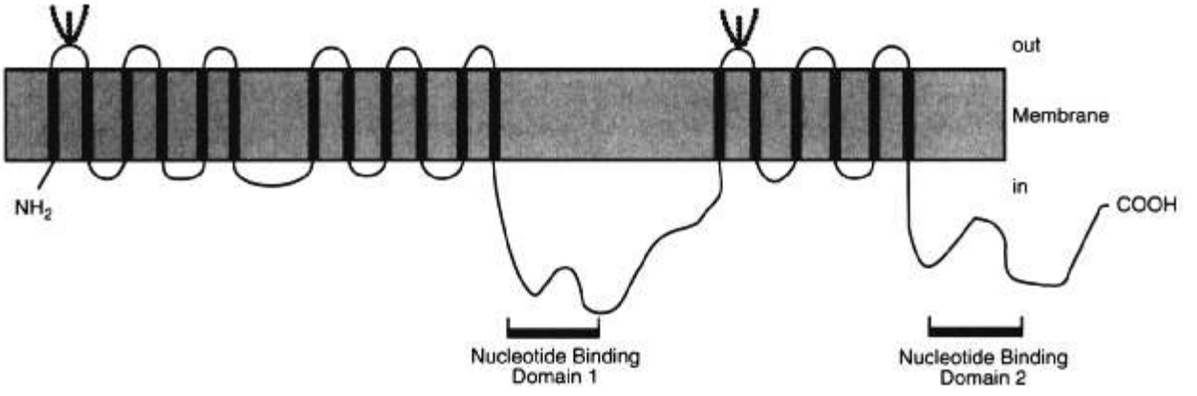

Figure 1. Schematic representation of proposed structure of MRP. Bold lines represent the transmembrane domains, while closed circles represent two potential extracellular *N*-linked glycosylation sites. Adapted from Stride *et al.*[25] and Loe *et al.*[26]

gene amplification is often present in laboratory-derived MDR cell lines, this is rarely observed in human tumor samples. Many investigations involving MRP have been performed at the RNA and protein level. A recent study using human lung cancer cell lines, non-small cell lung cancers and normal lung tissues found no amplification of *MRP*, despite high expression of this gene in a number of lung tumors.[34] Therefore, *MRP* gene amplification in human tumor samples is not likely to be a common mechanism of *MRP* overexpression.

Development of antibodies to MRP has aided in the elucidation of both the structure and expression of this protein in drug-resistant cell lines and tumor samples. A number of independent groups have prepared polyclonal antibodies against MRP which have contributed to the initial characterization of the cellular distribution and structure of this protein. Krishnamachary and Center[35] raised antibodies against synthetic peptides corresponding to various regions of the deduced sequence of the MRP protein. These antibodies recognized a 190 kDa protein present in the membranes of drug-resistant (HL60/ADR) cells, but not sensitive cells (HL60). In addition, Grant *et al.*,[15] using a polyclonal antibody against an MRP synthetic peptide, reported increased reactivity of a 190 kDa protein that correlated with increased resistance in HeLa cells transfected with the MRP gene. Using a different approach, Zaman *et al.*[22] raised antibodies to MRP by immunizing animals with a fusion protein of MRP. Development of monoclonal antibodies (mAbs) against MRP has provided a useful probe for the analysis of this protein using techniques such as immunoblotting, immunoprecipitation, immunofluorescence and flow cytometry.[36,37]

Phenotypic characterization of MRP

The MRP phenotype is characterized by resistance of cells to a range of MDR drugs, not dissimilar to those observed for P-glycoprotein-mediated MDR. These include the anthracyclines, *Vinca* alkaloids and epidophyllotoxins but, unlike P-glycoprotein-mediated resistance, it confers only low level resistance to taxol and colchicine.[19,22,38] MRP-expressing drug-selected cells usually display an MDR phenotype that is usually, but not always, related to reduced drug accumulation as a consequence of increased drug efflux.[26] Support for *MRP* as a putative drug resistance gene was obtained by transfection of the *MRP* gene into drug-sensitive HeLa cells.[15] Transfected cells expressed increased levels of *MRP* mRNA and MRP protein, and were resistant to vincristine and VP-16, but not to the non-MDR-associated drug cisplatin. Kruh *et al.*,[21] using a cDNA library transfer technique, found that drug-sensitive cells transfected with the gene obtained from drug-resistant (non-P-glycoprotein expressing) cells led to increased expression of the *MRP* gene and conferred a drug-resistance phenotype to these cells.

Cell lines which overexpress MRP usually display reduced drug accumulation and increased efflux which is ATP-dependent.[19,22,38] The biochemical basis of this mechanism has not been definitively established. Initial studies of both human and murine drug-resistant MRP-overexpressing cells demonstrated that increased levels of resistance correlate with increased levels of MRP protein.[19,33,35] While *MRP*-transfected cells also express increased levels of MRP protein, their relative cross-resistance levels often differ from drug-selected *MRP*-expressing cell lines.[15] For example, *MRP*-transfected cells had at least a 70-fold increase in *MRP* mRNA compared to the endogenous levels seen in parental cells, with a concomitant 15-fold increase in drug resistance.[15] In contrast, multidrug-resistant H69AR cells which express levels of *MRP* mRNA 100-fold above those of the parental drug-sensitive cells are between 50- and 100-fold resistant to doxorubicin compared to the parent cells. One possible explanation for this difference is that laboratory-derived drug-resistant cell lines, such as the H69AR cells, often display multiple alterations which may be contributing to the drug-resistance phenotype of these cells.[39] In addition, differences in the processing efficiency between the *MRP* harboring vector and the endogenous *MRP* mRNA may be contributing to this anomaly. An alternate explanation could be that the MRP may not function in isolation and transfection of only one component of a complex or dimer confers partial resistance to cells.

Variations in the distribution of MRP protein in both *MRP*-transfected and *MRP*-expressing drug-selected cells has been observed.[22,28,35,36,38] Localization of MRP to the plasma membrane has been reported by a number of groups, in both MRP-overexpressing and -transfected cells.[28,36,37] MRP protein has been localized to membrane fractions, predominantly in the endoplasmic reticulum and to a lesser extent in plasma membranes, in adriamycin-selected HL-60 cells.[35] In non-small cell lung cancer cells transfected with a vector harboring the MRP gene, *MRP* antibody staining was demonstrated in plasma membranes.[22] In contrast, NIH-3T3 *MRP*-

transfected cells were found to have an intracellular distribution of drug initially at the perinuclear fraction, followed by a punctate distribution of drug across the cytoplasm.[38] This pattern of drug distribution is consistent with the original hypothesis[14] that MRP may be involved in drug sequestration and perhaps vesicular transport. Differences observed in the subcellular localization of MRP may be dependent on variations in the intracellular trafficking of this protein in different cell types.

A number of groups have demonstrated that MRP functions as an ATP-dependent glutathione *S*-conjugate carrier (GS-X pump).[20,40,41] MRP has been shown to transport a number of reduced glutathione *S*-conjugates (GSH), including the cysteinyl leukotriene, LTC_4.[20,40–42] An inhibitor of LTC_4 transport, MK571, and an MRP mAb were found to inhibit LTC_4 photolabeling of a 190 kDa protein in MRP-expressing cells, indicating a role for MRP in cysteinyl leukotriene transport.[41,42] LTC_4, as are other cysteinyl leukotrienes, is a powerful intermediary of inflammation (increases vascular permeability) and smooth muscle contraction (reviewed in Keppler[43]). MRP, by its ability to transport hydrophobic anionic compounds in an ATP-dependent manner, bears a strong similarity to the recently identified multispecific anion transporter (MOAT).[44]

While the role of MRP in conferring drug resistance has been established, mechanisms responsible for this phenotype are not entirely understood. Using photoactive analogs of MRP-associated drugs, vinblastine and doxorubicin, no photoaffinity labeling of MRP could be demonstrated.[19,45] In addition, MRP-overexpressing drug-resistant cells were found to increase their drug accumulation, or decrease their drug efflux, following treatment with buthionine sulfoximine (BSO), an agent that depletes GSH.[46,47] Moreover, evidence that membrane vesicles are capable of pumping glutathione conjugates[20,40,41] has led to the hypothesis that MRP transports glutathione conjugates or anionic metabolites of drugs, and not unmodified chemotherapeutic drugs.[48] This is supported by a recent report that MRP transports glutathione, glucuronate and sulfated conjugates, but not unmodified doxorubicin, daunorubicin or vinblastine.[49] In contrast, another group using inside-out membrane vesicles isolated from MRP-transfected cells demonstrated that *MRP* is capable of transporting unmodified lipophilic chemotheraputic drugs.[50] Transport of daunorubicin also was inhibited competitively by drug–glutathione conjugates, as well as the LTC_4 antagonist MK571, suggesting a role for MRP transport of both conjugated and unmodified drugs. Recently, Loe *et al.*[42] demonstrated that MRP can indeed transport vincristine in an ATP-dependent manner but only in the presence of GSH.

The MRP/GS-X pump has recently been shown to transport cisplatin–glutathione complexes.[51] In this study, *MRP* gene expression was found to be higher in human leukemic cells selected for resistance to cisplatin, HL-60/R-CP, than in sensitive cells. Reduction of *MRP* mRNA levels was achieved by growing cells in the absence of cisplatin, in contrast to treatment with cisplatin and heavy metals which induced these levels to rise. Increased *MRP* mRNA correlated with increased activity of ATP-dependent transport of LTC_4, but not cisplatin. In addition to the induction of the MRP/GS-X pump by cisplatin, there was a concomitant increase in γ-glutamylcysteine synthetase, a rate-limiting enzyme in GSH synthesis, suggesting a role for this coordinated increase in resistance to cisplatin, heavy metals and alkylating agents. So why is overexpression of MRP not usually associated with cisplatin resistance? At present, the answer is not clear. One possibility is that formation of a cisplatin–glutathione complex is essential for the extrusion of cisplatin via the MRP/GS-X pump which, in turn, would be dependent on cellular GSH levels. Cells described by Ishikawa and colleagues[51] were selected for resistance to cisplatin, while others have examined MRP-transfected cells or cells selected for resistance to MDR-associated chemotherapeutic drugs. Tumor cells selected for resistance to chemotherapeutic agents often display multiple mechanisms of drug resistance.[52] Furthermore, induction of MRP expression in cisplatin-selected cells may not necessarily mirror that of MRP-expressing cells selected for resistance to an MDR-associated drug.

While data concerning the transport of chemotherapeutic drugs is at this stage conflicting, there are a number of points to consider. To date, there is no convincing evidence that drug conjugates are readily formed in tumor cells, particularly to account for the rapid drug efflux levels seen in some MRP-overexpressing cells.[22,38,47] During transport of arsenite from MRP-overexpressing cells, an increase export of glutathione has been reported.[47] Arsenite, unlike many commonly used chemotherapeutic agents belonging to the MDR phenotype, is readily conjugated. Partial depletion of glutathione levels in tumor cells can inhibit MRP-mediated transport of drugs, but does not affect the transport of calcein, an organic anion.[46] One hypothesis to explain some of these various findings is that the MRP requires glutathione to maintain its conformational state, which then enables the transport of these often

bulky large neutral or positively charged molecules.[53]

Modulation of MRP

Identification of MRP as an ATP-dependent drug efflux pump[14] led to the search for agents that could reverse this phenotype. A number of compounds can modulate drug distribution and sensitivity in MRP-overexpressing cells to various levels (refer to Table 1). The extent of modulation with the compounds listed in Table 1 varies with the cell type and selecting drug. In MRP-expressing cells, many of these agents afford only partial reversal of MDR. Verapamil and cyclosporin A are strong modulators of P-glycoprotein-mediated MDR, while in MRP-expressing cells they are not as potent.[19,22,38] This suggests that the action of these MDR modulators is not the same for MRP. Reversal of MRP-mediated MDR has been demonstrated with the tyrosine kinase inhibitor genistein.[64] The high concentrations required to modulate MRP-mediated MDR in these cells is unlikely to make this a clinically useful reversing agent. It is difficult to predict which agents will be effective modulators since they do not share distinct structural features.

MRP transport of cysteinyl leukotrienes has led investigators to examine agents that can reduce cellular levels of GSH and ultimately modulate the MRP-mediated MDR phenotype. One such agent is BSO, a potent inhibitor of γ-glutamylcysteine synthetase, an important enzyme in the GSH synthesis pathway.[66] BSO is a useful modulator of MRP-mediated MDR, particularly for daunorubicin, vincristine and rhodamine.[46,47,63] It is important to be cautious when interpreting the effect that an agent such as BSO has on MRP-mediated MDR. Depletion of glutathione leads to multiple cellular changes that may be influencing drug alterations in MRP-expressing cells.[67] The mechanism whereby BSO influences sensitization of cells to certain chemotherapeutic agents has not been determined.

MRP is a phosphoprotein, whose phosphorylation is likely to be regulated by a number of kinases. Phosphorylation of murine P-glycoprotein has been demonstrated using both protein kinase C (PKC) and protein kinase A.[68] In addition, human P-glycoprotein is also phosphorylated via the action of PKC and addition of PKC inhibitors appears to modulate MDR in cells expressing this membrane bound glycoprotein.[69] However, recent evidence suggests that phosphorylation/dephosporylation mechanisms are not essential for the function of P-glycoprotein-mediated resistance.[70] Addition of PKC inhibitors to human leukemia cells overexpressing MRP, results in reduced levels of MRP phosphorylation concomitant with an elevation in drug accumulation.[29] Inhibitors of PKC have been shown to mediate a number of non-specific effects in P-glycoprotein-mediated MDR cells (reviewed in Germann[30]) and a similar effect in MRP-overexpressing cells cannot be excluded. Elucidation of the phosphorylation sites on MRP and the role phosphorylation plays in the drug-resistance phenotype in MRP-overexpressing cells will help in the development of agents that can reverse this phenotype.

An alternate approach to the use of reversing compounds has been the use of antisense oligonucleotides. Inhibition of P-glycoprotein has previously been demonstrated using antisense oligonucleotides.[71,72] Stewart *et al.*,[73] using a similar approach in *MRP*-transfected cells, observed a decrease in the MRP with a concomitant increase in doxorubicin sensitivity. With improvements in the delivery of antisense oligonucleotide technology, this approach may one day prove valuable in reversing both MRP- and P-glycoprotein-mediated MDR.

Table 1. Agents that modulate the MRP-mediated MDR phenotype

Modulating agent	Class of agent	Reference
Verapamil	calcium channel blocker	54–57
Nicardipine and NIK250	calcium channel blocker	54, 56, 58
Cyclosporin A and PSC-833	immunosuppressants	59–61
GF109203X	PKC inhibitor	62
MK571	LTD_4 receptor antagonist	63
Genistein	tyrosine kinase inhibitor	64
Difloxacin	quinolone antimicrobial agent	65
Amiodarone	coronary vasodilator	61

MRP expression in tumors

Characterization of *MRP* as a drug-resistance gene in laboratory-derived drug-selected cell lines has been followed by considerable interest in the expression of this gene in both normal tissues and tumors. While the physiological role of MRP is not known, a wide range distribution of MRP has been detected in various tissues and tumors at both the RNA[31,74–76]

and protein[77] level. The broad pattern of expression suggests that MRP is required to perform a function that is common to many cell types. In 16 normal human tissues examined, low *MRP* mRNA expression levels were detected in brain, liver, small intestine and colon, while moderate to high levels were detected in lung, skeletal muscle, testis, ovary and peripheral leukocytes.[75] Essentially similar results were obtained with Western blotting and immunohistochemistry.[77]

Despite evidence that the MRP confers the MDR phenotype,[15,21] its clinical relevance is far from clear. *MRP* gene expression has been reported in hematological malignancies[74,78–83] and solid tumors.[34,84–89] The clinical relevance of MRP expression has been identified in only a small number of malignancies. In a subtype of acute myeloblastic leukemia, inversion of chromosome 16 is associated with a more favorable prognosis.[18] This inversion often results in deletion of the *MRP* gene. Daunomycin is commonly used in the treatment of this type of leukemia and reduction in MRP levels may contribute to increased sensitivity to this drug. Chronic lymphocytic and prolymphocytic leukemia's have been associated with high levels of *MRP* gene expression, while in acute myeloid leukemia levels are similar to normal hemopoetic cells with occasional high expressing samples (reviewed in Nooter *et al.*[83]). Bordow *et al.*[84] demonstrated that increased levels of the *MRP* gene correlated with amplification and overexpression of the N-*myc* oncogene in primary neuroblastoma tumors. This finding is of interest since amplification of the N-*myc* oncogene in neuroblastoma is a negative prognostic indicator, and is associated with a more aggressive and chemoresistant phenotype.[90] Furthermore, high-level *MRP* expression was associated with a poor outcome in neuroblastoma.[91]

Difficulty exists in the interpretation of many studies performed on clinical samples for expression of MRP, since a variety of techniques have been utilized. Few methods have been developed to determine whether MRP detected in tumor samples has a functional role. While uptake of fluorescent lipophilic dyes has been reported,[59,92] many of these substrates do not distinguish between P-glycoprotein- and MRP-expressing cells. Specific substrates that detect MRP and not P-glycoprotein are required. Flow cytometry techniques have proven useful for the detection of P-glycoprotein in hematological malignancies,[93] but, as yet, no mAbs against surface epitopes of MRP have been described. Analysis of tissues and tumors using RNA isolation-based techniques or Western blotting should be interpreted with caution as they not take into account the heterogeneity of tumors and MRP expression in various cell types.[77] Standardization of various techniques that have been recommended for P-glycoprotein-mediated MDR have also been proposed for MRP-mediated MDR.[53,93]

Conclusions

Characterization of the MRP-mediated MDR phenotype is progressing at a rapid rate. Many investigators are focusing their efforts on identifying normal physiological substrates for MRP, which will ultimately contribute to the development of appropriate agents to reverse this phenotype. In spite of the fact that MRP has been shown to function as a GS-X pump, evidence suggesting that it is capable of transporting unmodified drugs is presently limited. Whether this protein functions on its own or is part of a more complex system has not been determined. Mechanisms mediating multidrug resistance in the clinic are complex and diverse. The clinical relevance of increased levels of MRP is poorly understood and prospective controlled treatment studies relating MRP expression with clinical outcome are required to further our understanding of the role this protein plays in clinical MDR.

Acknowledgments

The author would like to thank Drs C-PH Yang and SB Horwitz and Ms CA Burkhart for helpful suggestions and critical reading of manuscript.

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(Received 24 October 1996; accepted 29 October 1996)