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Biology of the Multidrug Resistance-associated Protein, MRP

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INTRODUCTION

Resistance to multiple drugs is frequently encountered during treatment of many types of cancer by chemotherapy. Such resistance may develop during drug treatment, as with small cell lung cancer, or may be an inherent feature of a particular tumour type (e.g. non-small cell lung carcinoma or colon cancer) [1]. Multidrug resistance is often studied in the laboratory by using cultured cell lines as model systems. When drug-resistant cell lines are derived in the laboratory by exposure to a single chemotherapeutic agent, they frequently become crossresistant to many structurally and functionally unrelated compounds to which they have not previously been exposed. The spectrum of drugs encompassed by this form of multidrug resistance typically includes several classes of natural product drugs and related congeners, examples of which include the anthracyclines, vinca alkaloids and epipodophyllotoxins. However, unlike clinical multidrug resistance, the *in vitro* multidrug resistance phenotype does not usually include agents such as platinum-containing compounds, alkylating agents or antimetabolites [2–6].

To date, multidrug resistance in model systems is known to be conferred by two different integral membrane proteins, the 170 kDa P-glycoprotein (Pgp) [7–10] and the 190-kDa multidrug resistance-associated protein (MRP) [11–16]. These proteins belong to the ATP-binding cassette (ABC) [17] or traffic ATPase [18] superfamily of transport proteins, but their primary structures are quite dissimilar, sharing only approximately 15% amino acid identity. Nevertheless, MRP and Pgp confer resistance to a similar profile of chemotherapeutic agents [4, 13, 19, 20]. Since the procedures for deriving MRP- or Pgp-overexpressing cell lines by drug selection are similar, it is not clear what determines the preferential overexpression of either MRP or Pgp. However, overexpression of one protein does not preclude overexpression of the other, since a number of cell lines have recently been derived that overexpress both Pgp and MRP [21–23]. This review summarises current data on the structural and functional characteristics of MRP, its ability to confer multidrug resistance *in vitro* and its clinical relevance in drug-resistant malignant disease.

DISCOVERY OF MRP

Pgp overexpression has been associated with multidrug resistance in many drug-selected cell lines (reviewed in [24]) and some human tumours [25, 26]. However, there are now many examples of multidrug-resistant cell lines and tumours where Pgp is not involved. One of the most extensively characterised non-Pgp multidrug-resistant cell line is H69AR, a small cell lung cancer cell line which was derived from the drug-sensitive parental H69 line by step-wise selection in doxorubicin [27]. Although a number of biochemical alterations occurred during selection, including reduced levels of DNA topoisomerase II α and β [28, 29], increased expression of annexin II [30], a decrease in reduced glutathione (GSH), and altered levels of GSH-associated drug detoxification enzymes [31], none of these observed changes could completely account for the multidrug resistance phenotype of this cell line. Consequently, a search was undertaken for evidence of other alterations in gene expression using a differential hybridisation approach to identify mRNA species that are overexpressed in H69AR cells relative to parental H69 cells. Using this technique, a 6.5-kb mRNA was identified which was expressed in the resistant cells at levels approximately 100-fold higher than in the sensitive parental cell line. Sequencing of cDNA clones derived from this mRNA revealed that it had the potential to encode a 1531 amino acid protein that was predicted to be a member of the ABC transporter superfamily and was subsequently named the multidrug resistance protein, or MRP [11, 12, 16].

Since MRP overexpression in H69AR was associated with amplification of its cognate gene [11, 32], it was possible that the *MRP* gene was simply coamplified with the gene(s) that caused multidrug resistance. This possibility was excluded in two ways: (1) the continuous growth of H69AR in the absence of drug yielded a revertant line, H69PR, with decreased resistance to anthracyclines, vinca alkaloids and epipodophyllotoxins [30] and reduced levels of MRP mRNA [11]; and (2) the transfection of an MRP expression vector into Hela cells [13], demonstrated conclusively that the protein conferred resistance to the aforementioned classes of chemotherapeutic agents. The latter observation was subsequently confirmed in two other laboratories [14, 15].

Since its discovery in the human small cell lung cancer cell line H69AR, MRP has been identified in non-Pgp multidrug resistant cell lines from a variety of tumour types, including leukaemias, fibrosarcoma, and non-small cell lung, other small cell lung, breast, cervix, prostate, and bladder carcinomas (Table 1). Although many of these cell lines have been selected in doxorubicin or other anthracycline, others have been selec-

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Table 1. MRP expression in drug-selected tumour cell lines

Tumour type	Cell line	Selecting drug	[Ref.]
Human			
Small cell lung	H69AR	Doxorubicin	[11]
	GLC_4/ADR	Doxorubicin	[105]
	POGB/DX	Doxorubicin	[119]
	H69/VP	VP-16	[21]
	UMCC-VP	VP-16	[173]
Non-small cell lung	MOR/R	Doxorubicin	[174]
	CORL23/R		
Cervical	HeLa/J2	Doxorubicin	[11]
	KB/C-A	Doxorubicin*	[175]
	KB/7d	VP-16	[176]
Fibrosarcoma	HT1080/DR4	Doxorubicin	[32]
Leukaemia	HL60/ADR	Doxorubicin	[51]
	U-937/A	Doxorubicin	[22]
	CEM/E	Epirubicin	[142]
Breast	MCF7/VP	VP-16	[177]
	$MCF7/GL^R$	Geldanamycin	[178]
Bladder	T24/ADM	Doxorubicin	[23]
	KK47/ADM	Doxorubicin	
	5637/DR5.5	Doxorubicin	[179]
Prostate	P/VP20	VP-16	[143]
Mouse			
Erythroleukaemia	PC-V	Vincristine	[67]
	WEH1-3B/NOVO	Novobiocin	[180]

*With cepharanthine and mezerein.

ted in etoposide (VP-16) or vincristine. Recently, cell lines have been identified which co-overexpress both MRP and Pgp [21–23], but the relative contribution of each protein to the overall multidrug resistance phenotype has yet to be determined.

BIOCHEMICAL CHARACTERISATION OF MRP

Proteins belonging to the ABC superfamily of transport proteins are structurally diverse, but they all contain at least one hydrophobic polytopic transmembrane region and a cytoplasmic nucleotide binding domain (NBD) characterised by several signature motifs [33], with preferential binding to ATP [34]. Numerous eukaryotic and prokaryotic ABC proteins have been described which are all involved in the transport of molecules or ions across cellular membranes [17, 35]. There is broad variation in the chemical nature of the molecules transported by ABC proteins and, in mammalian cells alone, they range from chloride ions (transported by the cystic fibrosis transmembrane conductance regulator (CFTR) [36, 37]) to antigenic peptides associated with the endoplasmic reticulum of immune cells (TAP1/TAP2) [38, 39].

Most of the sequence similarity between MRP and Pgp is found within the NBDs which are generally conserved among members of the ABC superfamily. Computer-assisted hydropathy analyses suggest that MRP has a secondary structure distinct from that proposed for Pgp and a number of other eukaryotic ABC proteins (i.e. a tandemly duplicated molecule with six transmembrane segments and a NBD in each half [40]). The original topology proposed for MRP contained eight transmembrane domains in the NH_2-proximal half of the molecule and four in the COOH-proximal half, with both halves of MRP predicted to be *N*-glycosylated (Figure 1a) [11, 12]. The model we have developed most recently contains 11 or 12 transmembrane segments in the NH_2-proximal half, five or six of which are located in the first 230 amino acids of the protein, and six transmembrane segments in the COOH-proximal half (Figure 1b). Like the first model, both halves are predicted to be *N*-glycosylated. This model is based on alignment of hydropathy profiles of human and murine MRP, the Pgps, and the recently identified sulphonylurea receptor (SUR) [41, 42]. SUR, but not the Pgps, shares with MRP the additional five or six predicted NH_2-proximal transmembrane regions [43]. Members of the ABC superfamily with which MRP has the greatest degree of sequence identity include CFTR (19% amino acid identity), which mediates chloride conductance in airway epithelia and other tissues [36, 44, 45], rat SUR (29% amino acid identity) [41, 42], ItpgpA (32% amino acid identity), a *Leishmania* protein which confers resistance to arsenic and antimony-centred oxyanions [46, 47], YORI (33% amino acid identity), a yeast protein that mediates oligomycin resistance [48], and YCF1 (43% amino acid identity), a yeast protein that mediates cadmium resistance [49]. One distinguishing feature of MRP and the aforementioned ABC transporters (except for SUR) is the absence of 13 amino acids present between the Walker A and B motifs of NBD1 of the Pgps. While the 13-amino acid deletion is conserved among evolutionarily distant members of the MRP branch of the ABC superfamily, its functional importance is not known. Another distinguishing feature of MRP, YCF1, YOR1, Itpgp*A*, SUR and CFTR is the greater degree of primary sequence divergence between the first and second NBDs of these six proteins than between the two NBDs of the Pgps. These features, combined with differences in the number of transmembrane segments, sug-

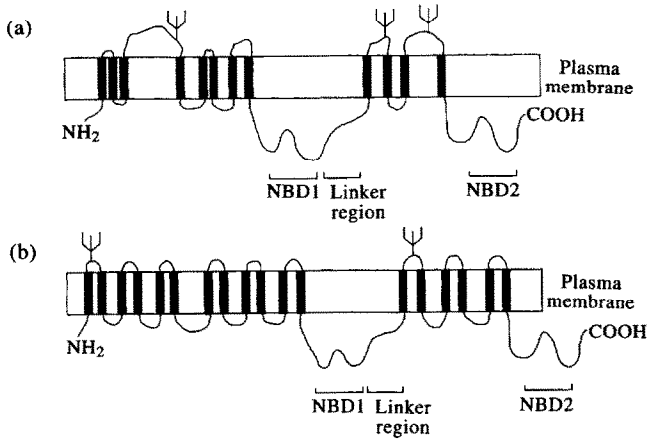


Figure 1. Models of multidrug resistance protein (MRP) membrane topology. MRP possesses features common to all members of the ATP-binding cassete (ABC) transporter superfamily, in that each half of the protein is predicted to consist of several transmembrane domains, followed by a cytosolic nucleotide binding domain (NBD). Current evidence indicates that MRP, unlike other ABC proteins such as the P-glycoproteins (Pgps), does not have a tandemly duplicated topology. The first model (a) is based on computer-assisted hydropathy analyses of the human MRP amino acid sequence and predicts that MRP is composed of 12 transmembrane domains (solid bars), eight of which are within the NH_2-proximal half of the protein [11]. According to this model, there are three potential sites for *N*-glycosylation in the extracellular regions of MRP. The second model (b) is based on a comparison of human and murine MRP with other ABC transporters [43], and suggests that there are up to four additional transmembrane domains in the NH_2-proximal half of the protein. According to this model, two potential extracellular *N*-glycosylation sites are predicted. Recent experimental studies on sites of glycosylation lend more support for the model shown in (b) (unpublished observations).

gest that MRP and Pgp may confer multidrug resistance by different mechanisms, as will be discussed later.

On the basis of its deduced amino acid sequence, MRP was predicted to be an ATP-binding, integral membrane *N*-glycosylated phosphoprotein with a calculated polypeptide molecular weight of 171 kDa. Qualitatively, the post-translational modifications and ATP-binding activity of MRP, which are implied from its primary structure, have been experimentally demonstrated [50–53]. MRP has been detected immunologically as a 190-kDa *N*-glycosylated phosphoprotein in H69AR and MRP-transfected HeLa cells and it can be labelled with a photoaffinity analogue of ATP [50]. Pulse chase studies on the processing of MRP indicate that it takes approximately 90 min to process the newly synthesised 170-kDa MRP polypeptide to the mature *N*-glycosylated 190-kDa form. The mature protein has a half-life of approximately 20 h in both cell types which is within the range typical of plasma membrane proteins subject to internalisation by endocytosis [54] and similar to that observed for Pgp [55, 56]. In both transfected and drug-selected cells, more than half of the newly synthesised MRP is not processed into mature protein and may be rapidly degraded in the endoplasmic reticulum, as observed with CFTR [57, 58]. Whether this also occurs during MRP biosynthesis in normal cells and tissues is not yet known.

Human MRP contains 12 potential sites (Asn-X-Ser/Thr) for N-linked glycosylation. However, the number of these which face the lumen of the endoplasmic reticulum and are accessible for glycosylation, as determined by the membrane topology of the protein (Figure 1a,b), has not been established experimentally. We have now confirmed that MRP is *N*-glycosylated in both halves of the molecule with complex oligosaccharides, which places some restrictions on the predicted topology of the protein (unpublished observations). The fact that the oligosaccharides are complex also indicates that the majority of the mature 190-kDa protein has been processed through the Golgi apparatus [50]. The functional significance of MRP *N*-glycosylation is unclear, since MRP-transfected cells treated with an inhibitor of *N*-glycosylation retain the ability to maintain reduced steady-state levels of cell-associated drug [50]. However, it remains possible that glycosylation influences MRP processing and stability.

Protein phosphorylation has been demonstrated to be involved in the regulation of some ABC transporters such as CFTR [59, 60] and possibly Pgp [61, 62]. MRP contains multiple sequence motifs that suggest the protein could be a target for several different kinases [11]. We and others have shown that MRP is phosphorylated *in vivo*, primarily on serine residues [50, 52, 63], but the specific amino acids in MRP that are phosphorylated and the kinases responsible have not been identified. Incubation of the human leukaemia HL-60/ADR cell line with the protein kinase C inhibitors staurosporine, H-7 or chelerythrine has been reported to result in reduced levels of MRP phosphorylation along with increased drug accumulation [52]. However, some protein kinase C inhibitors may interact non-specifically with other cellular components and, as has been shown for Pgp, may be substrates for transport [64]. These experiments were also carried out in drug-selected rather than MRP-transfected cells, and consequently, the observations cannot be attributed unequivocally to MRP overexpression. In preliminary studies with MRP-transfected cells, we have been unable to demonstrate an effect of staurosporine on drug accumulation (unpublished observations). *In vitro* analyses using membrane vesicle preparations, together with studies in which potential phosphorylation sites of MRP have been altered by site-directed mutagenesis will probably be necessary to establish firmly whether or not phosphorylation plays a role in MRP function.

MRP GENE STRUCTURE AND REGULATION

The human *MRP* gene has been mapped to chromosome 16 at band p13.13-13.12 [11, 32, 65]. It is amplified relatively frequently in drug-selected human cell lines that overexpress MRP mRNA [11, 32, 66, 67]. However, the cytogenetic alterations associated with amplification vary considerably between cell lines. In H69AR cells, for example, fluorescence *in situ* hybridisation analysis has demonstrated that multiple copies of the *MRP* gene are associated with large homogeneously staining regions (hsrs) on chromosomes other than 16 and with smaller hsrs on chromosomes 1, 6 and 7. The *MRP* gene is also amplified in double minute chromosomes (dms) that range in number from 2 to 100 per cell [32]. In contrast, in a multidrug-resistant fibrosarcoma cell line, HT1080/DR4, *MRP* is amplified at its normal location [32]. In another drug-selected cell line in which *MRP* is amplified, GLC4/ADR, copies of moderately repeated chromosome 16-specific DNA sequences have also been detected in dms (double minutes) and chromosomal hsrs [66]. It is possible that chromosome-specific repetitive sequences near the *MRP*

gene may contribute to the frequency with which *MRP* is amplified.

Evidence already exists that upregulation of MRP expression in drug-selected cells can occur by mechanisms other than gene amplification. Levels of MRP mRNA in the revertant H69PR cell line are several fold higher than in parental H69 cells, despite the fact that the former has retained only one copy of the *MRP* gene [11, 32]. In addition, elevated levels of MRP mRNA have been detected in the human drug resistant lung cancer cell line, SW-1573-30.3 and in murine myeloid leukaemia cell lines without an increase in *MRP* copy number [66, 67]. It is possible that *cis*-acting mutations of regulatory elements or changes in the expression of *trans*-acting factors in these cell lines increase transcription of the *MRP* gene. Alternatively, the stability of MRP mRNA may be altered. At present, little is known about the processes regulating transcription of the *MRP* gene or the degradation of MRP mRNA. The *MRP* promoter is extremely GC-rich and similar to promoters of many so-called housekeeping genes in that it lacks both TATA and CAAT motifs [68, 69]. As might be expected, sequence analysis of the 5′ flanking region of the gene reveals potential binding sites for many known *trans*-acting factors, such as Sp1, that could be involved in determining basal level of expression of the gene or in modulating its activity in response to a variety of stimuli. However, currently the functional significance of these sites has not been directly demonstrated.

The region encoding the 5′ untranslated leader sequence of MRP mRNA is also exceptionally GC-rich and contains a GCC triplet repeat [68, 69]. Such repeats are often polymorphic and this appears to be so with the MRP repeat. In H69, H69AR, H69PR and normal lymphocyte DNA, the triplet repeat consists of 13–14 copies [68], while MRP mRNA from HL60/ADR cells has only seven copies [69]. Whether this sequence has any influence on transcription, mRNA stability or translational efficiency remains to be determined. However, it is of interest to note that more extensive expansion of similar repeats has been implicated in the formation of fragile sites at several chromosomal loci and in the aetiology of several inherited disorders such as Huntington's disease [70]. It is currently unknown whether there are any human disorders that are attributable to an MRP defect, but the production of MRP knock-out mice should provide clues about this possibility.

PHYSIOLOGICAL RELEVANCE OF MRP

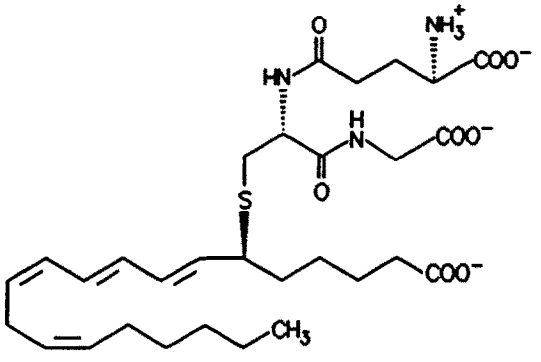
It has recently been demonstrated that MRP can transport the cysteinyl leukotriene, LTC_4 and some other GSH conjugates [20, 71–73] (Figure 2). LTC_4 is an arachidonic acid derivative involved in a number of receptor-mediated signal transduction pathways controlling vascular permeability and smooth muscle contraction [74]. It is synthesised from the precursor LTA_4 by conjugation with GSH, a reaction catalysed by the microsomal enzyme LTC_4 synthase [EC 2.5.1.37] [75]. After synthesis, it is exported from the cell in an ATP-dependent manner and converted extracellularly by the sequential action of the ectoenzymes γ-glutamyl-transpeptidase and LTD_4 dipeptidase [74] to the cysteinyl leukotrienes, LTD_4 and LTE_4, respectively.

Together, LTC_4, LTD_4 and LTE_4 make up the slow-reacting substance of anaphylaxis and play an important role in the pathogenesis of human bronchial asthma [76]. ATP-dependent transport of LTC_4, LTD_4 and LTE_4 by membrane vesicles prepared from MRP-transfected cells, together with observations that an LTD_4 cysteinyl leukotriene (CLT_1) receptor antagonist (MK571) [71] and a MRP-specific monoclonal antibody [73] suppress the photoaffinity binding of LTC_4 to MRP, have provided compelling evidence for the involvement of MRP in cysteinyl leukotriene transport.

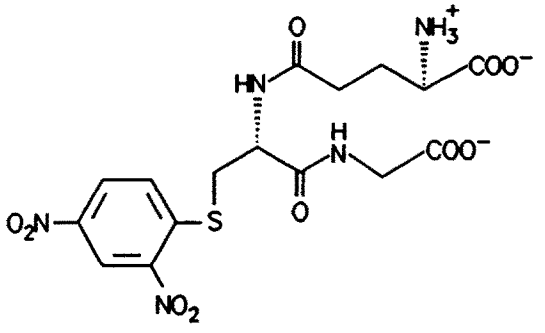
The ability of MRP to transport some other GSH conjugates [20, 71, 72] has prompted speculation that MRP may be a GSH conjugate and/or an organic anion transporter [77, 78]. Such transporters have been characterised functionally in a number of tissues and exhibit many transport characteristics similar to those of MRP [79–83]. MRP also confers resistance to heavy metal oxyanions in addition to chemotherapeutic drugs [19]. It is well known that GSH forms covalent complexes with heavy metal oxyanions [84, 85] and it is possible that such complexes may behave as MRP substrates. More recently, MRP has been shown to transport oxidised glutathione (GSSG) [86], as well as certain steroid glucuronides and bile salt derivatives [87] (Figure 2). Thus, it appears that MRP is able to transport a broad spectrum of hydrophobic anionic substrates, although the affinity of MRP for LTC_4 is higher than for any other compound examined thus far [71, 73, 86, 87].

It has been noted that the spectrum of hydrophobic anionic compounds which undergo MRP-mediated ATP-dependent transport is similar in some respects to those attributed to the multispecific organic anion transporter (MOAT) in liver canalicular membranes and other tissues [77, 79]. A specific protein responsible for this activity has not yet been identified, but the transport activity is functionally defined by the hepatocanalicular transport efficiencies observed in the TR^- rat [88, 89]. These include defects in transport of a number of conjugated anions, including the cysteinyl leukotrienes and glucuronides of bilirubin and thyroxine [90–92]. Many of the molecules whose transport is defective in the TR^- rat have at least two negative charges [93], a feature which is not shared by the steroid glucuronides which are transported by MRP [87]. Nevertheless, it has been proposed that MRP may be MOAT. Alternatively, MRP may be one of several transporters which contribute to MOAT activity in various tissues [77]. In support of this possibility, a novel member of the MRP branch of the ABC transporter superfamily has been detected which may also contribute to MOAT activity and organic anion secretion into bile [77, 94]. It is also possible that MRP may also transport peptides, at least those that are farnesylated. Very recently, MRP has been shown to complement the *STE6* mating mutation in *Saccharomyces cerevisiae* [95] and to be more effective at doing so than Pgp [96]. Thus, it appears likely that the farnesylated peptide, a mating factor, which is the physiological substrate for STE6, possesses additional structural determinants recognised by MRP.

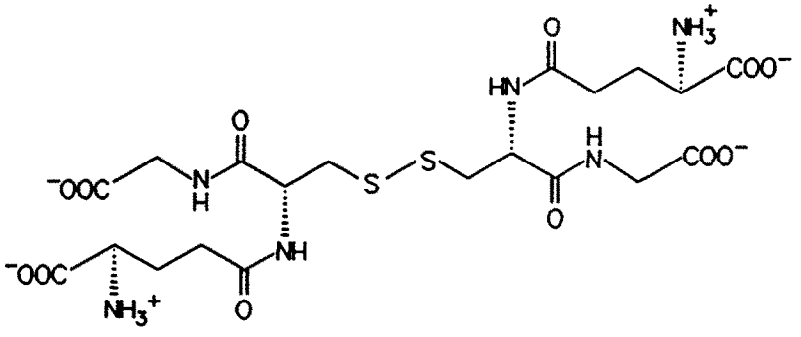
In addition to its function as a transporter, MRP may be involved in the regulation of endogenous channels and possibly other transporters. Several studies have demonstrated that ABC transporters can modulate the activity of ion channels, including outwardly rectifying Cl^- channels (CFTR) [97, 98], volume-regulated Cl^- channels (Pgp) [99, 100], and inwardly rectifying K^+ channels (SUR) [101]. We have also found that Cl^- and K^+ channel activity is increased in H69AR cells relative to the drug-sensitive parental cells [102, 103]. The recent demonstration that defects in the *SUR* gene result in aberrant regulation of K^+ channels in the β-cells of the pan-



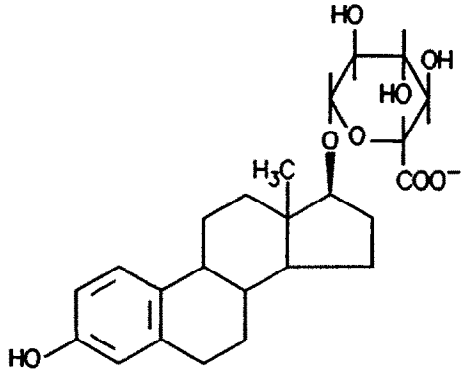
Leukotriene C_4



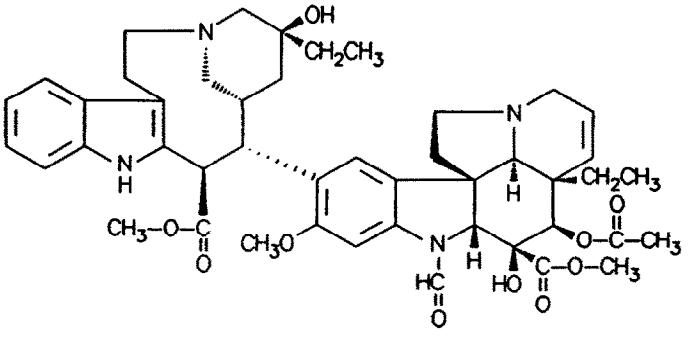
S-(2,4-dinitrophenyl)glutathione



Glutathione disulphide (GSSG)



17β-oestradiol 17-(β-D-glucuronide)



Vincristine*

Figure 2. Chemical structures of some known multidrug resistance protein (MRP) substrates. Studies of ATP-dependent transport by membrane vesicles derived from MRP-transfected cells have demonstrated direct active transport of the anionic compounds shown. In contrast, although MRP is known to confer resistance to a broad spectrum of natural product drugs, direct MRP-mediated transport of these agents has not been demonstrated. However, very re(ently, MRP-mediated ATP-dependent transport of vincristine has been shown, but only in the presence of physiological concentrations of glutathione. Current data suggest that LTC_4 is the highest affinity substrate for MRP (K_m 97–105 nM) [71, 73], followed by 17β-estradiol 17-(β-D-glucuronide) (K_m 2.5 μM) [87] and GSSG (K_m 93 μM) [86]. No kinetic parameters for S-(2,4-dinitrophenyl)GSH transport have been reported, but at a single substrate concentration (50 nM), the rate of transport by membrane vesicles derived from MRP-transfected HeLa cells was 16% of that measured for LTC_4 [71]. *Transport of this compound is observed only in the presence of GSH [73].

creas, together with the level of amino acid identity between the two proteins (29%), suggests the possibility that MRP may function as a channel regulator. However, the mechanism by which MRP may alter channel activity, and its physiological relevance, are as yet unknown.

MRP mRNA is expressed at low levels in many tissues, including haematopoietic cells [11, 104, 105]. Analyses of the tissue distribution of mRNAs for human and murine MRP indicate that the highest levels of expression are in testes, skeletal muscle, heart, kidney and lung [11, 43, 105, 106]. MRP mRNA is also readily detectable in brain and spleen with the lowest relative levels being present in liver and intestine [43]. At present, the specific cell types in which *MRP* mRNA is expressed are not known for most tissues. *In situ* hybridisation studies in the mouse lung indicate that the highest levels of expression are in the bronchial epithelium [43]. In mouse testis, stage-specific MRP mRNA expression has been detected in haploid spermatids [43]. In the liver, MRP may be specifically targeted to hepatocanalicular and lateral membranes [77, 94]. The physiological functions of MRP in these tissues is not known, nor has the relative amount of functional MRP protein been determined. Several MRP-specific monoclonal antibodies have been developed [107, 108] and should be useful in determining the spatial pattern of MRP expression in a wide range of tissues. The results of biochemical studies and knowledge of the cell types in which MRP is expressed should provide useful clues to its physiological role(s). A powerful complementary approach will be to generate mice in which the *MRP* gene has been "knocked out". Such mice will provide *in vivo* models not only for elucidating the physiological function of MRP, but also for determining the possible clinical consequences of blocking its function. Our recent cloning and characterisation of murine *MRP* mRNA represents the first step in these important experiments [43].

MECHANISTIC ASPECTS OF MRP-MEDIATED MULTIDRUG RESISTANCE

MRP confers a pattern of drug resistance similar to that of the resistance-conferring Pgps, but there may be some differences. For example, studies to date indicate that MRP confers only low levels of resistance to paclitaxel and colchicine [14, 19, 109], which are reported to be among the best "substrates" for Pgp [110, 111]. Another notable difference is the ability of MRP to confer low levels of resistance to arsenic and antimony-centred oxyanions [19]. Functional analysis of Pgp has shown that it acts as an ATP-dependent drug efflux pump, and this has been experimentally demonstrated by direct measurement of the uptake of unmodified drug (e.g. vinblastine and colchicine) into plasma membrane vesicles or proteoliposomes [112–115].

Reduced drug accumulation and enhanced drug efflux is usually observed in drug-selected cells that overexpress MRP [16, 116], as it is with cells overexpressing Pgp [2]. However, variation in the relative levels of crossresistance to certain chemotherapeutic agents is exhibited by different drug-selected and MRP-transfected cell lines, the mechanistic basis of which is not understood. In MRP transfectants, ATP-dependent reduced drug accumulation and enhanced drug efflux are observed [14, 19, 109]. Altered intracellular distribution of anthracyclines has been detected in several drug-selected MRP-overexpressing cell lines, and in some cases, shown to be energy dependent [6, 22, 117–125]. This has led to the hypothesis that MRP may participate in sequestering drugs away from their cellular target [11]. Differences in the subcellular distribution of MRP have also been detected in drug-selected and in certain transfected cells [50, 51, 108]. In some cell lines, MRP has been reported to be predominantly in the endoplasmic reticulum [51] while in others, including SW1573 [14] and MRP-transfected HeLa [50] cells, the protein was found predominantly on the plasma membrane or in post-Golgi vesicles [108, 126]. What governs these apparent differences in subcellular localisation is unknown, but cell-type dependent variations in trafficking of MRP may be involved. Interestingly, H69AR cells, which express very high levels of MRP, also overexpress annexin II which is involved in the trafficking of endosomal membranes [30, 127, 128]. Although there is as yet no evidence that MRP is directly involved in the vesicular sequestration of drugs, it is possible that differences in distribution of MRP between various membrane compartments may contribute to the variation in drug accumulation and efflux kinetics observed in different MRP-overexpressing cell lines.

The mechanism by which MRP confers resistance to multiple drugs is not well-understood. Pgp can be labelled by photoactive analogues of cytotoxic agents, suggesting that certain natural product drugs can bind to Pgp without modification [129]. In contrast, it has not been possible to photolabel MRP with photoaffinity analogues of vinblastine or doxorubicin and ATP-dependent transport of unmodified drug by itself has not been demonstrated [19, 130]. These findings, combined with identification of several conjugated molecules as potential physiological substrates, have led to the speculation that while MRP does not appear to transport unmodified drugs directly, it may instead transport drug conjugates. However, there is no evidence in tumour cells that conjugation to GSH or other endogenous small molecules (e.g. glucuronic acid) are important pathways for biotransformation of chemotherapeutic agents to which MRP confers resistance [131]. Moreover, there is little data indicating that these processes occur to any significant extent in extrahepatic tissues or drug-resistant cell lines.

Very recently, using vincristine as a model substrate, we have shown that ATP-dependent uptake of unmodified drug by membrane vesicles derived from MRP-transfected HeLa cells can be demonstrated, but only in the presence of GSH [73] (Figure 2). The mechanism by which GSH-dependent vincristine transport occurs is unknown, but it is consistent with the observation that increased drug accumulation occurs in some MRP-overexpressing cells following depletion of GSH [132]. It is possible that GSH (or perhaps other organic anions such as glucuronate) interact directly with MRP and this interaction is necessary for transport. Alternatively, vincristine and GSH may spontaneously form a complex which behaves as a MRP substrate. However, evidence of GSH cotransport by MRP is equivocal [132, 133]. Experiments designed to address the mechanistic aspects of GSH-dependent drug transport by MRP, as well as the structural determinants which are critical for MRP recognition, are in progress.

CHEMOSENSITISATION OF MRP-ASSOCIATED MULTIDRUG RESISTANCE

Chemosensitisers are a structurally diverse group of compounds that are able to reverse the multidrug resistance phenotype and hence are of interest because of their potential to circumvent multidrug resistance clinically [134, 135].

Many studies have analysed the structural features of chemosensitisers that reverse Pgp-mediated multidrug resistance, but less is known about reversal of MRP-associated multidrug resistance. In some instances, compounds which reverse resistance have been shown to bind to Pgp, suggesting that they interact directly with the protein and interfere with its function [136]. Chemosensitisers such as verapamil and trifluoperazine are also among the most effective stimulators of the ATPase activity associated with Pgp [137]. However, most of these agents are usually much less effective at reversing resistance in non-Pgp multidrug resistant cell lines, many of which are now known to overexpress MRP [4, 118, 138, 139]. This is perhaps not surprising given the different substrate specificities exhibited by the two transporters. Furthermore, MRP could not be labelled with a photoaffinity analogue of verapamil in HL60/ADR cells [130], suggesting that, in contrast to Pgp, any sensitising effect of verapamil does not involve direct interaction with MRP.

Compounds reported to increase drug accumulation, alter drug distribution and/or modulate resistance in MRP-overexpressing cells at least to some degree, include the phenylalkylamine calcium channel blocker verapamil [119, 138, 140–142], the dihydropyridines nicardipine [138] and NIK250 [140, 143], the tiapamil analogue DMDP [4], the bisindolylmaleimide protein kinase C inhibitor GF109203X [144], the anionic quinoline LTD_4 receptor (CLT_2) antagonist MK571 [145], the cyclosporin analog PSC 833 [118, 146], the isoflavanoid tyrosine kinase inhibitor genistein [147], the quinolone difloxacin [148], and the diiodinated benzofuran amiodarone [146]. As is the case for Pgp, there appear to be no conserved structural features which predict the ability of a compound to reverse MRP-associated multidrug resistance *a priori*. Some of these compounds restore accumulation only at concentrations that are highly toxic which precludes their use *in vivo*, and are unable to restore completely drug accumulation or drug sensitivity to levels comparable with sensitive cells. Moreover, the extent to which resistance to different drugs is reversed can vary significantly between cell lines and occasionally within the same cell line. This variability is not entirely surprising, given that most studies to date have been carried out using drug-selected cell lines, which may have undergone multiple alterations contributing to resistance. Consequently, it is not possible to attribute the effects of potential reversing agents exclusively to their action on MRP. Only a few studies have been carried out on MRP-transfected cells and these suggest that agents such as verapamil and cyclosporin A do not specifically inhibit the action of MRP [14, 19, 109], consistent with previous observations in MRP-overexpressing drug-selected cell lines.

The ability of MRP-enriched membrane vesicles to transport the cysteinyl leukotrienes has led to the suggestion that compounds which alter cellular GSH levels may modulate MRP-associated resistance. The most common agent used experimentally to deplete GSH in cultured cells is buthionine sulphoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase which is the rate-limiting enzyme of the GSH biosynthetic pathway [149]. Although BSO did not alter DOX resistance in H69AR cells [31], it has been reported to enhance anthracycline and/or vincristine sensitivity in certain MRP overexpressing cell lines [132, 145, 150–152], including transfected HeLa cells (unpublished observations). Thus, as with other chemosensitising agents described above, there is some variation in the effects of BSO in different model systems, which is not yet understood. Depletion of GSH could have a variety of effects on signal transduction pathways that may influence MRP-mediated resistance [153, 154]. It remains to be determined whether BSO acts as a sensitiser by decreasing formation of drug–GSH complexes, by impeding GSH-dependent transport of unmodified drug, or by some as yet unidentified mechanism. Studies in transfected cells and reconstituted *in vitro* systems are in progress that should elucidate the relationship between GSH levels and MRP mediated resistance to certain drugs.

An attractive alternative to using small molecules as "conventional" chemosensitisers is to employ antisense oligonucleotides, because of their potentially high degree of specificity. Rather than inhibiting protein function, antisense oligonucleotides inhibit protein synthesis by binding specifically to complementary nucleic acid targets [155, 156]. We have investigated this approach by assessing the relative abilities of 15 phosphorothioate oligonucleotides to reduce MRP mRNA and protein levels. We identified a sequence, ISIS 7597, complementary to nucleotides 2107-2126 of MRP that reduces levels of MRP mRNA and protein in transfected HeLa cells by $> 90\%$ and 70%, respectively [157]. Cells treated with ISIS 7597 showed enhanced sensitivity to doxorubicin. We have also shown that this reduction in MRP mRNA is probably mediated by RNase H cleavage at the site of ISIS 7597 hybridisation. While the efficacy of ISIS 7597 has not yet been demonstrated *in vivo*, the *in vitro* results affirm the potential of antisense oligonucleotides for reversing MRP-mediated resistance.

CLINICAL RELEVANCE AND RESPONSIVENESS TO CHEMOTHERAPY

Despite the relatively recent discovery of MRP, the evaluation of its importance as a prognostic indicator and as a target for chemotherapeutic agents or chemosensitisers is progressing rapidly. Experimentation in this area will be facilitated by the availability of reagents and methods for the sensitive and specific detection of MRP mRNA [104, 158, 159] and protein [107, 108]. To date, most studies have examined tissue levels of MRP mRNA by Northern blot analysis [11], reverse transcriptase/polymerase chain reaction (RT-PCR) [158, 159], or RNase protection assays [105]. However, caution must be exercised in the interpretation of data obtained by these methods, as they provide no indication of the heterogeneity of MRP expression between specific cell types of a given tissue or its subcellular distribution. Furthermore, it is possible that the amount of functional MRP protein does not always correlate with levels of MRP mRNA [13]. The availability of immunoreagents for the detection of MRP should allow for a comprehensive examination of its tissue and cell-type distribution, as well as its clinical relevance in tumours [160–162].

It should be borne in mind that immunodetection of MRP may not necessarily be indicative of the amount of functional protein. Thus, the development of a specific and sensitive functional assay for MRP is clearly indicated. The utility of measuring MRP activity by monitoring uptake of fluorescent lipophilic dyes has been explored [163, 164] (unpublished observations). An analogous approach has already been developed for measuring Pgp activity since active extrusion of several fluorescent dyes has been demonstrated *in vitro* for this transporter [165, 166]. Cellular accumulation, distribution and efflux of dyes can be conveniently measured in live cells

Table 2. MRP expression in human tumours

Tumour type	MRP expression	Test	[Ref.]
Leukaemias			
Acute myeloid	+	mRNA	[181]
Chronic lymphocytic	+		
Acute myeloid	+	mRNA	[182]
B-chronic lymphocytic	+	mRNA	[183]
B-prolymphocytic	+		
Hairy cell	−		
Non-Hodgkin's	−		
Myeloma	−		
Acute myeloid	−	mRNA	[104]
Myeloma			
B-chronic lymphocytic	+	mRNA	[184]
Acute lymphoblastic	+	mRNA	[185]
Acute myeloid	−		
Acute myeloid	−	DNA	[65]
Acute myeloid	±	mRNA	[186]
Acute lymphocytic	+	mRNA	[161]
Chronic lymphocytic	±		
Prolymphocytic	+	mRNA	[187]
Chronic lymphocytic	+		
Acute myeloid	±		
Acute lymphocytic	±		
Hairy cell	−		
Non-Hodgkin's lymphoma	−		
Multiple myeloma	−		
Chronic myelocytic	−		
Lung (NSCLC)	+	mRNA	[188]
	±	mRNA and protein	[161]
Lung			
Squamous cell carcinoma			
Poorly differentiated	−	protein	[162]
Well differentiated	+		
Anaplastic thyroid	+	mRNA	[169]
Neuroblastoma	+	mRNA	[171]
Oesophageal squamous cell carcinoma	±	mRNA and protein	[161]
Soft tissue sarcoma	−		
Breast	−		
Kidney	−		
Ovary	−		
Colon	−		
Melanoma	−		
Prostate	−	mRNA	[161]
Bladder	−		
Testis	−		

by flow cytometry, confocal microscopy or spectrofluorimetry. The acetoxymethylester of calcein has been shown to undergo energy-dependent efflux from MRP-overexpressing cell lines [167]. However, similar efflux kinetics have also been observed in cells overexpressing Pgp [166], which limits the specificity of the assay. Therefore, more specific substrates for MRP are clearly required before this approach will be useful for assessing the clinical relevance of MRP in drug-resistant tumours. The recent identification of MRP as an ATP-dependent transporter for cysteinyl leukotrienes and certain other GSH conjugates suggests that this activity may be useful for screening MRP activity in certain tissues and tumour biopsies. However, the technical difficulties involved with membrane vesicle transport assays precludes their use for general screening. Moreover, GSH–conjugate transport activity may not be solely attributable to MRP in all tissues [168].

High levels of MRP mRNA have been detected in some tumour cell lines that characteristically respond poorly to

chemotherapy, examples of which include non-small cell lung [162] and thyroid [169] carcinomas, gliomas [170] and neuroblastomas [171]. Several studies involving the step-wise selection of a series of drug-resistant lines often exhibit the overexpression of MRP in low level resistant cells, followed by Pgp overexpression in the same cells at higher levels of resistance [21–23]. These observations suggest that elevated MRP expression may be more likely to occur at lower, clinically relevant levels of drug resistance.

MRP mRNA and in some instances the protein have also been detected in malignant cells from a variety of tumour types (Table 2). MRP is frequently expressed in several forms of leukaemia. An interesting exception is provided by some cases of the M4Eo subclass of acute myeloid leukaemia, in which a part of one *MRP* allele has been deleted [65]. In a preliminary study, this deletion was associated with markedly increased duration of disease-free survival following initial therapy [65]. It is possible that a reduction of MRP levels may increase sensitivity to daunomycin, which is commonly used to treat this form of leukaemia and to which MRP is known to confer resistance [19]. While less is known about the expression of MRP in solid tumours, preliminary studies indicate that levels of MRP are elevated in neuroblastoma [171]. Interestingly, this study also demonstrated a significant correlation between the levels of MRP in primary tumours and amplification of *N-MYC*, which is currently the strongest negative prognostic indicator for response to chemotherapy for neuroblastoma patients [172]. These data suggest a relationship between the cellular processes involved in tumour progression and those involved in resistance to chemotherapeutic agents.

CONCLUSION

Our understanding of the biological properties of MRP has progressed rapidly since it was identified in the H69AR cell line in 1992. It is now firmly established that MRP can confer a multidrug resistance phenotype that *in vitro* is similar in many respects to that conferred by Pgp. Insufficient data are available to assess whether or not these two proteins play a similar role *in vivo* in drug resistant malignancies but the normal physiological functions of these two proteins clearly differ. This, together with the lack of structural similarity between MRP and Pgp, makes their common ability to confer multidrug resistance somewhat surprising. The fact that they can, raises the distinct possibility that other as yet unidentified members of the ABC superfamily may also be involved in multidrug resistant disease.

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