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COMMENTARY

Multidrug Resistance Mediated by the Multidrug Resistance Protein (*MRP*) Gene

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ABSTRACT. Inherent or acquired resistance to multiple natural product drugs is a major obstacle to the success of chemotherapy. Two proteins have been shown to cause this type of multidrug resistance in human tumour cells, the 170 kDa P-glycoprotein and the 190 kDa multidrug resistance protein (MRP). Overexpression of these N-glycosylated phosphoproteins in mammalian cells is associated with reduced drug accumulation. Both MRP and p-glycoprotein belong to the ATP-binding cassette superfamily of transmembrane transport proteins, but they share only 15% amino acid identity. Furthermore, their predicted membrane topologies differ considerably, with MRP containing three multispanning transmembrane domains compared with the two that are present in P-glycoprotein. The drug cross-resistance profiles of cells that overexpress MRP or P-glycoprotein are similar but not identical. For example, lower levels of taxol resistance are associated with overexpression of MRP than with overexpression of P-glycoprotein. There also appear to be fundamental differences in the mechanisms by which the two proteins transport chemotherapeutic drugs. P-glycoprotein-enriched membrane vesicles have been shown to directly transport several chemotherapeutic drugs, whereas vincristine transport by MRP-enriched membrane vesicles is demonstrable only in the presence of reduced glutathione. Several potential physiologic substrates of MRP including leukotriene C_4 and 17β-estradiol-17-(β-D-glucuronide) have been identified. In contrast, these conjugated organic anions are transported poorly, if at all, by P-glycoprotein. Finally, agents that reverse P-glycoprotein-associated resistance are usually much less effective in MRP-associated resistance. Antisense oligonucleotide-mediated suppression of MRP synthesis offers a highly specific alternative approach to circumventing resistance mediated by this novel drug resistance protein. BIOCHEM PHARMACOL 52;7:967–977, 1996.

KEY WORDS. multidrug resistance; transmembrane protein; vincristine; glutathione; leukotriene C_4; antisense oligonucleotides

Inherent or acquired MDR[‖] is a major barrier to the success of chemotherapy. *In vitro*, MDR appears after exposure of cells to a single drug, which is almost always a natural product, and is characterized by resistance to structurally unrelated compounds with different subcellular targets. Only two human proteins are presently known that can cause this form of multidrug resistance. The first to be identified was P-glycoprotein, a 170 kDa transmembrane phosphoglycoprotein [1, 2]. P-glycoprotein acts as an energy-dependent outward transport pump, removing drugs from the cytoplasm and/or directly from the plasma membrane, thereby decreasing intracellular drug accumulation. Overexpression of P-glycoprotein has been demonstrated to be a relatively common occurrence in some human tumours [3], but it does not explain all instances of MDR *in vivo* or in drug-selected cell lines characterized by lowered drug accumulation or increased drug efflux.

Recently, we cloned an mRNA encoding a second MRP from the MDR small cell lung cancer cell line H69AR [4]. Although H69AR cells have a phenotype similar to MDR cell lines that overexpress P-glycoprotein, they do not overexpress this transporter [5–7]. Instead, their MDR phenotype is now known to be associated primarily with elevated levels of 190 kDa MRP [4]. In this review, we will focus on the pharmacological properties of MRP-overexpressing cells and will discuss current knowledge concerning the role of MRP in normal and malignant cells.

MRP STRUCTURE

Computer-assisted analysis of the open reading frame of MRP mRNA suggested that it encoded a member of the ABC superfamily of transmembrane transporters [4, 8]. The

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‖ *Abbreviations*: MDR, multidrug resistance; MRP, multidrug resistance protein; ABC, ATP-binding cassette; NBD, nucleotide binding domain; MOAT, multispecific organic anion transporter; cMOAT, canalicular MOAT; PKC, protein kinase C; LT, leukotriene; GSH, glutathione; Rh123, rhodamine 123; BSO, buthionine sulfoximine; and pH_i, intracellular pH; MRP, multidrug resistance protein.

MRP polypeptide was predicted to be 171 kDa and, like many vertebrate members of the ABC superfamily, to contain two NBDs, each preceded by a multi-spanning transmembrane region. Initially, a membrane topology was proposed, based solely on hydropathy analyses of the predicted human MRP amino acid sequence, consisting of an NH_2-proximal region that spanned the membrane eight times and a COOH-proximal region that contained only four membrane-spanning helices [4]. More extensive comparative analyses of the hydropathy profiles of human MRP and its highly conserved murine homolog with those of several P-glycoproteins suggest that this model is incorrect [9]. The hydropathy profiles of the MRPs and P-glycoproteins, when aligned at their COOH termini, are very similar despite the fact that the amino acid sequence identity between the two proteins is very low (approximately 15%) and restricted to the cytoplasmic, generally conserved NBDs [4]. This suggests that the MRPs may have two transmembrane domains with topologies similar to those present in the P-glycoproteins, plus an NH_2-proximal hydrophobic domain of approximately 230 amino acids that contains up to six additional membrane-spanning helices (Fig. 1) [9]. Three recently identified ABC transporters may also have topologies similar to MRP. These include the β cell sulfonylurea receptor which is involved in regulating insulin secretion [10], the proposed MOAT defective in the congenitally hyperbilirubinemic TR^- rat [11], and the yeast cadmium resistance factor YCF1 [12].

The amino acid sequence of MRP contains variants of the so-called Walker motifs involved in ATP binding, as well as potential sites for a variety of post-translational modifications [4]. Biochemical studies using drug-selected cells and MRP-transfected cells have confirmed that MRP can bind ATP [13]. The protein has also been shown to be glycosylated and phosphorylated, primarily on serine residues [13, 14]. Pulse-chase experiments combined with glycosidase digestion have demonstrated that the unmodified MRP polypeptide has an apparent mass of 170 kDa and is processed into a mature 190 kDa form by the addition of N-linked complex oligosaccharides [13]. The topological model of human MRP (Fig. 1) predicts that only two or possibly three of its eleven potential glycosylation sites are external to the plasma membrane, depending on the precise positioning of the transmembrane helices. Regardless of whether two or three sites are, in fact, exposed on the cell surface, the model predicts that both NH_2- and COOH-proximal halves of the protein are glycosylated, and this has been confirmed experimentally (unpublished observations). The mature protein is relatively stable with a half-life of approximately 20 hr. However, a significant percentage of the protein produced in both drug-selected and transfected cells is degraded rapidly in the endoplasmic reticulum [13]. Whether this process is regulated or not is currently unknown. The precise location of the phosphorylated serine residues and their functional importance is also not known. In P-glycoprotein and cystic fibrosis transmembrane conductance regulator, phosphorylation occurs predominantly in the so-called "linker" region of these proteins [15–17], and this may also be true of MRP. The protein kinases responsible for the phosphorylation of MRP have not been identified, although preliminary inhibitor studies have suggested that PKC may be involved [14]. However, comparison of the human and murine MRP amino acid sequences through the linker region reveals only a low level of conservation of potential PKC phosphorylation sites between these two, otherwise highly conserved, homologs.

RESISTANCE PATTERNS ASSOCIATED WITH OVEREXPRESSION OF MRP

The MDR H69AR cells from which MRP was cloned exhibit a 50-fold resistance to doxorubicin, the drug used for their selection [5, 7]. H69AR cells are also resistant to a variety of other natural product drugs, many of which are lipophilic cations at physiologic pH [7, 18]. A similar pattern of resistance has been described in other cell lines overexpressing MRP and typically includes cross-resistance to anthracyclines, epipodophyllotoxins, and *Vinca* alkaloids, but not anti-metabolites or platinum-containing drugs. Although the resistance profiles of drug-selected MRP or P-glycoprotein-overexpressing cell lines are similar, demonstrable differences exist. These differences are also apparent when cells transfected with one or the other of the two proteins are compared. In contrast to P-glyco-

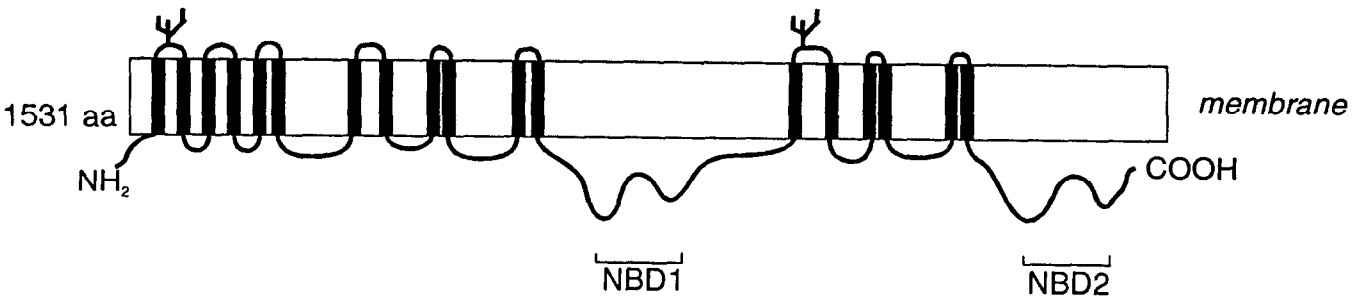

FIG. 1. A predicted secondary structure of MRP. Like all members of the ABC superfamily of transport proteins, each half of MRP is predicted to consist of several membrane-spanning segments, followed by a cytosolic NBD. The model shown is based on a comparison of human and murine MRP with several other ABC transporter proteins [9].

protein-overexpressing cells, MRP-transfected HeLa cells are resistant to certain heavy metal oxyanions (e.g. arsenite), but not to mitoxantrone, and they show only low level resistance to colchicine and taxol [19]. The resistance profile of murine NIH 3T3 cells transfected with human MRP is similar to that reported for transfected HeLa cells but taxol resistance was not observed [20]. MRP-transfected human SW1573 lung cancer cells also have a similar profile except that they are relatively more resistant to colchicine than the anthracyclines, and are not resistant to taxol or arsenite [21, 22]. Although it is possible that these differences may be attributable to the cell type used for transfection, they may also be explained by differences in the levels of MRP in the various transfectants.

Overexpression of either P-glycoprotein or MRP, or in some cases both protein, can clearly be caused by exposure to the same types of drugs. Although MRP-associated MDR has been observed most frequently after selection of cells with doxorubicin, MRP-overexpressing cells selected in VP-16, vincristine, epirubicin, and novobiocin have now been described [23], as well as cell lines expressing both proteins [24–26]. What determines whether MRP or P-glycoprotein will be overexpressed is not known. It has been suggested that overexpression of MRP may confer initial low levels of resistance, while overexpression of P-glycoprotein may appear later, in response to higher drug concentrations. In several studies, including those with human myeloid leukemia cells after doxorubicin selection [24], human lung cells after VP-16 selection [25], and bladder cells after doxorubicin selection [26], it has been shown that MRP is overexpressed early during selection in relatively low concentrations of drug. During subsequent selection in higher drug concentrations, expression of MRP may decrease or remain constant and overexpression of P-glycoprotein may emerge. However, this does not occur in all cases. For example, we found that repeated exposure of H69AR cells to higher concentrations of doxorubicin did not result in higher levels of resistance or in the overexpression of P-glycoprotein (unpublished observations). In contrast, parental H69 cells subjected to a similar selection protocol in a different laboratory resulted in a resistant cell line that overexpressed P-glycoprotein rather than MRP [27]. Whether these *in vitro* observations have implications for the development of acquired, clinical MDR remains to be established.

MRP AND DRUG ACCUMULATION, EFFLUX, AND DISTRIBUTION

Most drug-selected cells that overexpress MRP, including the leukemic cell line HL60/ADR and the lung cancer cell lines COR-L23/R and GLC4/ADR, show a decrease in drug accumulation accompanied by enhanced drug efflux [28–30]. However, in MRP-overexpressing MCF7/VP cells, decreased accumulation was not associated with a detectable increase in drug efflux [31]. Differences in the pharmacologic properties of drug-selected cells are difficult to interpret because of the possibility of multiple changes occurring during the selection process. This complication is diminished by using transfected cells, and MRP transfectants have been uniformly found to display a decrease in drug accumulation [19–21]. Whether reduced accumulation can be completely explained in all cases by an increase in efflux has not been firmly established.

MRP has been reported to be located predominantly in the plasma membrane of several cell lines including MRP-transfected HeLa and SW1573 cells, as well as the drug-selected cell lines H69AR, COR-L23/R, and GLC4/ADR [13, 32, 33]. The localization of MRP in the plasma membrane supports a role for this protein as an efflux pump. In HL60/ADR cells and certain other cells, however, MRP has been reported to be mainly in the endoplasmic reticulum or associated with cytoplasmic structures, possibly the Golgi apparatus [32, 34–36]. Thus, under some conditions and in some cell types, significant amounts of MRP appear to be located in intracellular membranes. Whether MRP in all of these intracellular compartments contributes to the drug resistance phenotype is unknown.

Digitized video fluorescence microscopy has been used to take advantage of the fluorescence properties of anthracyclines (daunorubicin, doxorubicin) to examine intracellular drug distribution. In drug-selected MRP-overexpressing cells, alterations in drug localization have been observed in addition to enhanced drug efflux. It has been found that in sensitive cells, these drugs localize to the nucleus and cytoplasm with a high nuclear/cytoplasmic ratio. In contrast, anthracyclines accumulate mainly in the perinuclear region or in cytoplasmic vesicles in resistant cells, and much less in the nucleus [34, 37, 38]. It should be borne in mind that alterations in drug distribution have also been reported in P-glycoprotein-overexpressing cells [39, 40], indicating that this property is not associated exclusively with overexpression of MRP. The perinuclear site could include the Golgi apparatus where maturation of proteins occurs, as suggested by colocalization studies with fluorescent ceramide derivatives specific for different organelles [41, 42]. In some cases, incubation of cells with ATP inhibitors, or at temperatures below 18°, restores normal cell distribution of drugs, indicating that sequestration is energy dependent [37, 43, 44]. Several compounds including brefeldin A and bafilomycin also restore nuclear accumulation of anthracyclines in some but not all resistant cells [34, 45]. However, it has not been possible to correlate redistribution of drug by these agents with enhanced sensitivity because these compounds restore accumulation only when used at toxic concentrations [45]. Consequently, the functional importance of these changes in drug distribution with respect to the drug resistance phenotype of these cells remains uncertain. Nevertheless, it is tempting to speculate that MRP located on intracellular membranes could be involved in this drug redistribution.

Although we have observed marked changes in daunorubicin distribution in drug-selected H69AR cells when

compared with parental H69 cells [37], we also found that patterns of distribution could be altered significantly by varying the drug concentration and incubation time, as reported by others [34, 38, 43]. Nevertheless, after exposure of H69 and H69AR cells to relatively low concentrations of daunorubicin (0.5 μM) (with and without washing, and with and without time for drug efflux), we found that the drug localized to the nucleus and the nuclear membranes in sensitive H69 cells as expected, but in H69AR cells fluorescence was primarily localized to the cytoplasm in a punctate pattern. However, no significant differences in daunorubicin distribution could be demonstrated in MRP-transfected HeLa cells although many different conditions were tested (unpublished observations). These observations suggest that cell type, level of MRP expression, or other ancillary changes in the drug-selected cells may contribute to the cytoplasmic sequestration observed in H69AR and possibly other MRP^+ cells. In contrast, Kruh and colleagues [20] transfected human MRP cDNA into murine NIH 3T3 cells and observed a punctate extranuclear pattern of daunorubicin fluorescence in the transfected cells compared with the nuclear fluorescence in control cells. The reason for the discordant findings between these two transfected cell populations is unclear, but may reflect differences between the cells into which the MRP DNA was transfected.

Intracellular pH

The anthracyclines and *Vinca* alkaloids are weak bases that are charged molecules in neutral and acidic environments, suggesting that changes in pH_i can alter the proportion of drug within the cell that is protonated. In their cationic form, the drugs are less able to diffuse across plasma or vesicular membranes. Consequently, pH_i has the potential to influence both drug accumulation and sequestration, and thus ultimately drug resistance [46, 47]. In some cases, the pH_i of drug-selected P-glycoprotein and non-P-glycoprotein-resistant cell lines has been found to differ from that of the respective drug-sensitive parental cell line [48–50]. To determine whether overexpression of MRP resulted in changes in pH_i, we carried out a series of flow cytometry experiments with the pH indicator dyes SNARF and BCECF. These studies revealed that although dye accumulation in both H69AR and MRP-transfected HeLa cells was reduced by 80–90% compared with their sensitive counterparts (Table 1), there were no significant differences in pH_i between resistant and sensitive cells (Fig. 2). These data are consistent with those of other investigators using MRP-transfected lung cells [21] and suggest that changes in the pH gradient across the plasma membrane do not make a significant contribution to MRP-mediated resistance. Presently, however, the possibility cannot be excluded that in certain cell types, MRP expressed on intracellular membranes could alter the pH gradient across the vesicular membrane(s) and in this way increase drug sequestration.

MRP TRANSPORT ACTIVITY

Transport of Endobiotic Substrates

There is now considerable evidence that MRP can function as a high affinity transporter of cysteinyl leukotrienes, most notably the GSH-conjugate LTC_4 [51–53]. This arachidonic acid derivative and its metabolites, LTD_4 and LTE_4, are active components of the "slow reacting substance of anaphylaxis." They are involved in the control of vascular permeability and smooth muscle contraction and have been implicated in the pathogenesis of asthma [54]. Initially, elevated levels of ATP-dependent transport of LTC_4 and certain other GSH conjugates were demonstrated in membrane vesicles from drug-selected HL60/ADR cells [55].

TABLE 1. Relative accumulation of fluorescent pH indicator dyes and calcein-AM in transfected (T5) and drug-selected (H69AR) MRP-overexpressing cells

	Relative accumulation*(%)			
Compound	**C1**	**T5**	**H69**	**H69AR**
SNARF-1-AM†	100 ± 14	3.4 ± 1.1	47.6 ± 9.4	4.6 ± 4.1
BCECF-AM‡	100 ± 33	11.7 ± 6.7	140 ± 64	26.9 ± 14.8
Calcein-AM	100 ± 25	12.4 ± 5.3	47.1 ± 17.6	9.1 ± 4.0

* Control transfected (C1) and MRP-transfected (T5) HeLa cells and parental H69 and doxorubicin-selected MRP-overexpressing H69AR cells (1×10^6 cells/mL) were incubated with calcein-AM (2 μM), SNARF-1-AM (5 μM), or BCECF-AM (2 μg/mL) for 30-40 min at 37°. Cells were washed (except for cells incubated with calcein-AM), and then analyzed with a Coulter Epics Elite flow cytometer at an excitation wavelength of 488nm. Fluorescence emitted from cells incubated with calcein-AM was measured through a 525 nm bandpass (20 nm bandwidth) filter. Fluorescence from cells incubated with SNARF-1-AM was measured through 575 nm bandpass (15 nm bandwidth) and 630 nm bandpass (5 nm bandwidth) filters. Fluorescence from cells incubated with BCECF-AM was measured through 525 nm bandpass (20 nm bandwidth) and 630 nm bandpass (5 nm bandwidth) filters. Ten thousand cells were counted, and data are expressed as a percentage of fluorescence in control transfected HeLa (C1) cells. The results shown are the means ± SD of values obtained in 3–5 independent experiments. All fluorescent compounds were obtained from Molecular Probes (Eugene, OR).

† 5- (and -6)-Carboxy SNARF®-1 acetoxymethylester.

‡ 2', 7'-Bis (2-carboxyethyl)-5 (and -6)-carboxyfluorescein acetoxymethylester.

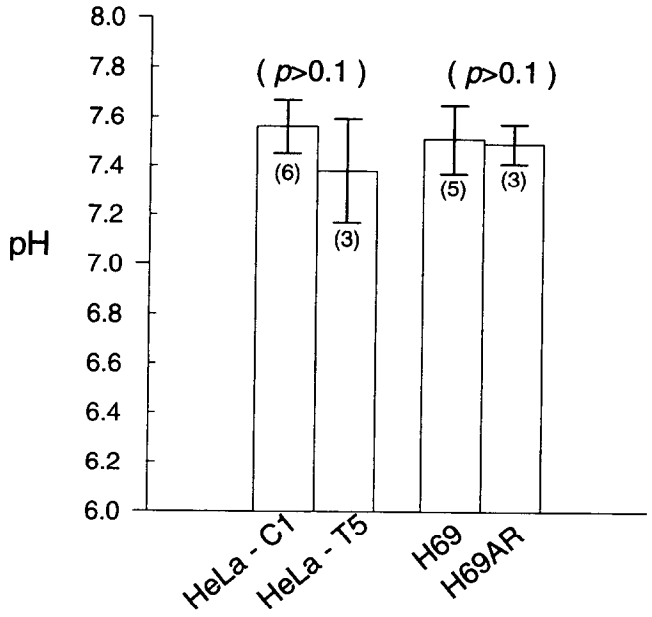

FIG. 2. Intracellular pH in MRP-overexpressing drug-selected (H69AR) and transfected HeLa (T5) cells and their sensitive parental cells. Cells (1 × 10^6/mL) were incubated with 5 μM SNARF®-1-AM (Molecular Probes, Eugene, OR) for 40 min at 37°, washed, and then analyzed on a Coulter Epics Elite flow cytometer. Fluorescence excitation was at 488 nm, and fluorescence emission was measured after passing through 575/15 and 630/5 bandpass filters. Ten thousand cells were counted, and pH was calculated from the ratio of 630/575 nm fluorescence using a calibration curve. This curve was generated by labelling cells with SNARF-1-AM followed by resuspension in high [K^+] buffers at different pHs ranging from 6.4 to 8.2 (increments of 0.2 pH units). After addition of nigericin (5 μg/mL) to equilibrate the pH inside and outside the cell, fluorescence ratios were determined and plotted against pH. Bars represent the means ± SD of values obtained in 3–6 independent experiments (indicated in parentheses).

[^{3}H]LTC_4 was also shown to photolabel a 190 kDa protein present at relatively high levels in these vesicles and labelling of the protein could be prevented by the LTD_4 receptor antagonist, MK571 [51]. The ability of MRP to bind and transport LTC_4 has been confirmed and extended using membrane vesicles derived from both drug-selected and MRP-transfected cells [51–53]. We have provided immunological proof that the 190 kDa protein photolabelled with [^{3}H]LTC_4 is MRP and have shown that both LTC_4 transport and photolabelling can be inhibited by the MRP-specific monoclonal antibody QCRL-3, which recognizes a conformation-dependent epitope of the protein [53, 56]. These studies indicate that direct binding of LTC_4 to MRP precedes transport.

Several other potential physiological substrates for MRP have also been identified by direct transport studies, among them glutathione disulfide (oxidized glutathione) and steroid glucuronides such as 17β-estradiol 17-(β-D glucuronide) [57, 58]. Many organic anions have been shown to act as competitive inhibitors of either LTC_4 or 17β-estradiol 17-(β-D glucuronide) transport, some of which are also candidates for physiological substrates. These include a wide range of anionic conjugates of bile salts and steroid hormones [57–59]. The broad substrate specificity of MRP for organic anions prompted the suggestion that MRP may be the MOAT. The function of this transporter had been studied extensively for a number of years, but it had not been characterized physically. In rodent liver, cMOAT mediates the biliary excretion of endogenous and exogenous compounds following their modification by Phase II conjugating enzymes to form amphiphilic anions. Hepatocanalicular MOAT has also been shown to be functionally defective in the congenitally conjugated hyperbilirubinemic TR^- rat [60].

Despite certain common pharmacologic features of MRP- and MOAT-mediated transport, several lines of evidence indicate that they are different proteins, the most compelling being the recent cloning of a novel canalicular ABC transporter from rat liver [11]. This cloned transporter has yet to be characterized functionally. However, it has been designated as cMOAT because it is expressed predominantly in the hepatocanalicular membrane, whereas the majority of hepatic MRP appears to be located basolaterally [61]. Thus, it appears likely that cMOAT may be involved in hepatocanalicular organic anion transport, whereas MRP may carry out a similar function in nonhepatic tissues and possibly basolateral membranes in hepatocytes.

Consistent with its proposed identity, the putative cMOAT mRNA in the TR^- rat contains a frameshift mutation that results in substantially reduced levels of the protein in hepatocanalicular membranes [11]. The size of cMOAT is similar to that of MRP, but its predicted amino acid sequence is only 48% identical. This level of identity is not much higher than that found between MRP (or cMOAT) and the yeast cadmium resistance protein YCF1, indicating a relatively ancient divergence of these two mammalian transporters when compared with that leading to diversification of mammalian P-glycoprotein isoforms.

Transport of Chemotherapeutic Drugs

Although MRP has been shown to reduce cellular accumulation of at least some of the drugs to which it confers resistance, such as daunorubicin, VP-16, and vincristine, it has not been possible to demonstrate direct transport of these compounds or other unmodified chemotherapeutic drugs by MRP-enriched membrane vesicles [52, 53, 58]. These chemotherapeutic agents have also been found to be very poor inhibitors of LTC_4 transport [52, 53]. Furthermore, photoactivatable analogs of doxorubicin and vinblastine that have been shown to label P-glycoprotein appear not to bind to MRP [19, 62]. These observations, combined with the demonstrated ability of MRP to transport cysteinyl leukotrienes, led to the suggestion that MRP reduced drug accumulation, not by transporting unmodified drugs directly, but by effluxing drugs after their conjugation to GSH. This hypothesis was attractive, in part, because it provided a plausible explanation for the broad cross-

resistance associated with overexpression of MRP. However, GSH conjugation is not known to be an important pathway for the biotransformation of chemotherapeutic agents to which MRP confers resistance and there is no evidence that this reaction occurs to any significant extent in drug-resistant tumor cell lines. Moreover, GS-conjugates of daunorubicin or vincristine are not detectable in the culture medium of MRP-overexpressing cells exposed to either of these two drugs [22]. Alternative possibilities currently under investigation are that MRP can co-transport GSH and drug without conjugate formation, or that GSH facilitates drug transport by an as yet undefined mechanism. Although oxidized GSH is transported by MRP, reduced GSH appears not to be a substrate [57], nor does it inhibit transport of other compounds [53]. However, we recently determined that the ability of vincristine and vinblastine to inhibit LTC_4 transport is enhanced markedly by the addition of physiological concentrations of GSH [53]. A similar enhancement was not observed with other reducing agents such as dithiothreitol, 2-mercaptoethanol, or L-cysteine, indicating a very specific requirement for GSH. Consistent with its effect on the inhibitory potency of the *Vinca* alkaloids, GSH also increased direct transport of vincristine in membrane vesicles derived from MRP-transfected cells [53]. In the absence of GSH, vincristine transport was extremely low and showed no ATP dependence. In the presence of increasing physiological concentrations of GSH, ATP-dependent vincristine transport was enhanced markedly in a concentration-dependent fashion [53]. It remains to be determined whether a co-transport mechanism is involved or whether GSH causes a change in MRP structure that facilitates drug binding and/or transport [53, 63].

Detection of MRP Transport Activity

The identification of MRP as a transport protein has prompted a search for substrates that will allow MRP activity to be measured in intact human tumour cells and to be distinguished from the transport activity of P-glycoprotein. However, no suitable MRP-specific substrate has yet been identified. P-glycoprotein can extrude a number of lipophilic fluorescent dyes commonly used in cell biology [64, 65]. Flow cytometric measurement of efflux of one of these dyes, Rh123, has become a popular method for assessing P-glycoprotein function, particularly in hematological malignancies [66–70]. For reasons that are not yet understood, it takes a remarkably long time to reach steady-state levels of this compound in cells that overexpress MRP. In our MRP-transfected HeLa cells, steady-state levels of Rh123 were not achieved at 1 hr, and at this time levels of accumulation did not differ significantly from those in control cells. A similar delay in achieving steady-state conditions and displaying differential Rh123 accumulation has been observed by others when comparing both drug-selected and transfected MRP-overexpressing cells with their drug-sensitive counterparts [63, 71, 71]. In contrast, efflux from MRP^+ cells preloaded with Rh123 is increased markedly compared with parental cells [71] (unpublished observations). Thus, while rapid establishment of steady-state levels of reduced Rh123 accumulation may be indicative of P-glycoprotein activity, increased efflux of this dye can be attributable to the activity of either transporter.

In contrast to the results obtained with Rh123, we have observed reduced accumulation of calcein acetoxymethylester (calcein-AM) in H69AR cells, as well as in MRP-transfected HeLa cells (Table 1). Others have also reported in several different MRP-overexpressing cell lines that calcein-AM retention is decreased and concluded that calcein-Am is a better substrate for MRP than Rh123 because it displays a larger and more rapid differential accumulation efflux [72–74]. However, calcein-AM is also a substrate for P-glycoprotein [65]. The lack of suitable dye substrates with which to distinguish the activities of MRP and P-glycoprotein has resulted in a search for compounds that can selectively inhibit the activity of one or the other transporter. Feller *et al.* [72, 73] tested several fluorescent compounds (Rh123, daunorubicin and calcein-AM) in combination with several potential inhibitors (PSC 833, vincristine, genistein) in a panel of drug-selected MRP^+ cell lines. On the basis of these and other studies, they have suggested that the Rh123 efflux in the presence and absence of cyclosporin A can be used to estimate P-glycoprotein activity and that daunorubicin efflux in the presence and absence of genistein can be used to provide an indication of MRP activity. They also found that the effect of PSC 833, which has been demonstrated previously to be an inhibitor of P-glycoprotein-mediated transport, varied markedly among the different MRP^+ cell lines [72]. On the other hand, the modulation of anthracycline accumulation by the tyrosine kinase inhibitor genistein was found to be rather specific for MRP-overexpressing cells [72, 75, 76]. However, the effect required high concentrations of genistein (>200 μM), which are generally quite toxic to many types of cells [76]. Furthermore, in our MRP-transfected HeLa cells, we found that genistein (100 μM) had no effect on either calcein-AM accumulation or R123 efflux (unpublished observations), and the effect of 200 μM genistein on daunorubicin accumulation in MRP-transfected SW1573 cells was also very small [72]. The mechanism by which high concentrations of genistein influence MRP activity is unclear, and it does not appear to be effective in all cell types.

REVERSAL OF MRP-ASSOCIATED RESISTANCE

MRP overexpression has been detected in a wide variety of human and mouse drug-selected cell lines from various tissues, as well as in non-selected human tumor cell lines, including those derived from small cell and non-small cell lung, colon, gastric, breast, and ovarian carcinomas, neuroblastoma, thyroid, and glioma [23, 77, 78]. Expression of MRP protein or its mRNA has also been detected in tumor samples from patients with chronic and acute leukemias and a variety of carcinomas from different tissue types [23,

26]. Because of its widespread occurrence in human tumors, there is considerable interest in identifying ways in which MRP-mediated resistance can be reversed.

One pharmacological approach to circumventing drug resistance is to employ agents that enhance the cytotoxicity of known antineoplastic agents (so-called modulators or chemosensitizers). While many compounds have been proven to be very efficient at inhibiting P-glycoprotein activity [79], few of these are as active on MRP. For example, verapamil and cyclosporin A, which are well characterized inhibitors of P-glycoprotein, usually show no, or only a small effect, on drug sensitivity and transport in drug-selected and MRP-transfected cells [7, 19, 21, 80–82]. Compounds reported to reverse MRP-associated resistance to a significant degree in at least one model system, include the dihydropyridines nicardipine and NIK 250 [80, 83], difloxacin [84], the bisindolymaleimide GF109203X [85], MK 571 [86], the tiapamil analog DMDP [7], and the cyclosporin analog PSC 833 [81]. Sensitization by these compounds is often dependent on the drug and type of cells tested. For example, in HL60/AR cells, GF109203X completely reversed vincristine resistance but has less effect on doxorubicin resistance whereas in GLC4/ADR cells, GF109203X only partially reverses vincristine resistance [85]. In contrast, we were unable to show any significant effect on this compound on either vincristine or doxorubicin resistance in MRP-transfected HeLa cells (Fig. 3).

Gekeler *et al.* [86] reported that the LTD_4 receptor antagonist MK571 also modulated resistance of HL60/AR and GLC4/ADR cells to vincristine and, to a lesser extent, doxorubicin. MK571 competes for LTC_4 binding and transport in MRP-enriched membrane vesicles [51]. However, the apparent sensitizing effect of this compound in intact cells is somewhat surprising because the LTD_4 receptor is expressed on the cell surface and MK571 is relatively hydrophilic with a limited ability to cross the plasma membrane. Finally, the isoflavanoid genistein, which has little or no effect in MRP-transfected cells, has been reported to inhibit daunorubicin efflux in several drug-selected MRP^+ cell lines including GLC4/ADR [75, 76]. However, this effect was observed only at toxic concentrations precluding the use of genistein as a reversing agent *in vitro* or *in vivo*.

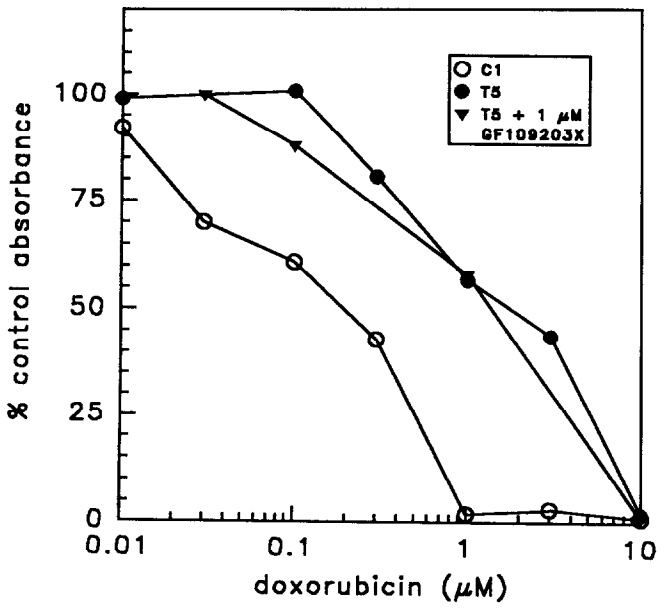

FIG. 3. Effect of GF109203X on doxorubicin resistance in MRP-transfected (T5) HeLa cells. Chemosensitivity was measured using the MTT assay [18, 87]. Cells were set up at 1 x 10^4 cells/well in microtiter plates on day 0. Doxorubicin and GF109203X (1 μM) were added on day 1, and cell viability was measured on day 5. Each point represents the mean of quadruplicate determinations in a typical experiment. Error bars have been omitted for clarity but SD were <10%.

The compounds reported to inhibit MRP-associated drug efflux or to at least partially reverse MRP-associated resistance share little or no structural similarity. As has been the case in the search for P-glycoprotein-reversing agents, it is difficult to propose a rational strategy for the synthesis of more specific inhibitors of MRP. Consequently, a large scale screening approach using MRP-transfected cells may be the most efficient way to identify reversing agents specific for this resistance protein.

Depletion of GSH

The observation that membrane vesicles from MRP-overexpressing cells could transport the glutathione conjugate LTC_4 suggested that intracellular GSH levels might be involved in MRP-associated drug resistance. The levels of GSH in transfected and drug-selected MRP^+ cells relative to their sensitive parental cells vary from being considerably lower in some (e.g., H69AR [88], COR-L23/R [63], MCF7/VP [89] and MRP-transfected HeLa T5 cells (Fig. 4), to being comparable (e.g. MRP-transfected SW1573 cells [22]) or significantly higher (e.g. GLC4/ADR cells [22, 63]) in others. To explore further a possible role for GSH, we and others have tested the γ-glutamylcysteine synthetase inhibitor BSO for its ability to reverse resistance in MRP-overexpressing cells. As with other "modulators," there is considerable variation in the amount of chemosensitization achieved in different model systems, and the basis of this variability is not understood. In some cells, sensitization by BSO is complete for certain chemotherapeutic drugs but not for others [22, 86, 88, 89, 91]. For example, in MRP-overexpressing drug-selected MCF7/VP cells, sensitivity to vincristine was completely restored by BSO, whereas the reversion was only partial for VP-16 resistance [89]. In H69AR cells, we found that BSO restored vincristine but not doxorubicin sensitivity, and similar results were obtained in MRP-transfected HeLa T5 cells ([88] and unpublished observations). In contrast, in HL60/AR cells, BSO restored sensitivity to both drugs [86, 92]. Zaman *et al.* [22] reported that BSO decreased daunorubicin efflux from MRP-transfected lung tumour cells but not from *MDR1*-transfected cells. However, BSO caused a significant increase in daunorubicin sensitivity in both resistant cell types. These results suggest that the BSO effect may not be

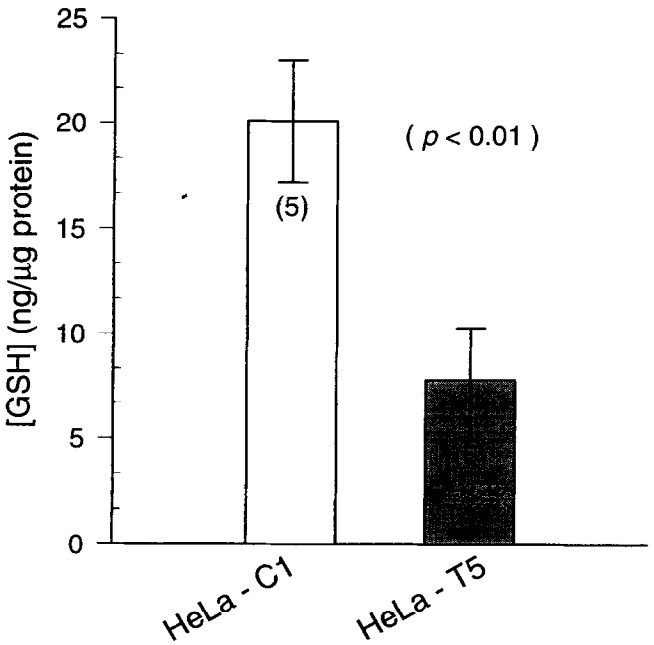

FIG. 4. Total GSH levels in MRP-transfected (T5) and control (C1) transfected HeLa cells. The levels of total oxidized and reduced GSH were determined by the spectrophotometric method of Tietze [90].

completely specific for MRP-associated resistance [93]. Moreover, depletion of GSH by BSO could have a variety of effects on signal transduction pathways that may indirectly influence MRP- or P-glycoprotein-mediated resistance. Thus, the precise mechanism by which BSO increases drug sensitivity in certain instances remains to be determined.

Alternative Approaches to Reversing Drug Resistance

One alternative approach to circumvention of MRP-mediated resistance is the use of antisense oligonucleotides or synthetic catalytic RNAs (ribozymes) to reduce MRP expression by targeting in a sequence specific manner the mRNA coding for MRP. These approaches avoid any dependence on protein structure and function and have been used with some success to reverse P-glycoprotein-mediated resistance *in vitro* [94–96]. We recently described a series of phosphorothioate oligonucleotides that reduce MRP mRNA and protein expression in MRP-transfected HeLa cells [97]. The decrease in MRP mRNA by one of these oligonucleotides, ISIS 7597, was shown to be very rapid and complete, and likely to result from RNase H-mediated cleavage of the mRNA. This was demonstrated by the detection of two appropriately sized degradative mRNA fragments from ISIS 7597-treated cells in northern blot analysis with two DNA probes specific to the 5′ and 3′ ends of MRP mRNA, respectively. However, the decreases in MRP mRNA and protein levels were short-lived, and only a partial sensitization to doxorubicin was observed [97]. Further investigation of MRP-specific oligonucleotides that have been modified chemically (e.g. at the 2′OH position of the sugar moiety) is ongoing. Such modified molecules may be more effective at reducing MRP expression for more prolonged periods of time and, consequently, be capable of more complete reversal of MRP-mediated resistance. Testing of these MRP-specific antisense oligonucleotides in animal models is also in progress.

CONCLUSION

At present, two proteins have been demonstrated unequivocally to cause MDR in human tumour cells, the 170 kDa P-glycoprotein and the 190 kDa MRP. While both proteins belong to the ABC superfamily of transport proteins, they are structurally quite distinct molecules. Increased levels of MRP have been found in cells displaying low levels of resistance, and in several instances its overexpression has been shown to precede that of P-glycoprotein during drug selection. This observation, combined with the fact that resistance is usually low in clinical samples compared with drug-selected cell lines, suggests that MRP may be more relevant in clinical drug resistance. The drug cross-resistance profiles of cells that overexpress MRP or P-glycoprotein are similar but clearly not identical. Furthermore, the potential physiologic substrates of MRP are transported poorly or not at all by P-glycoprotein. There also appear to be fundamental differences in the mechanisms by which the two proteins transport chemotherapeutic drugs. Finally, agents that can reverse one type of resistance are usually much less effective in the other. These differences in cross-resistance profiles, mechanism of action, and sensitivity to reversing agents represent a significant challenge in the design of treatment protocols effective against both drug-resistance proteins. They also serve to emphasize the importance of developing reliable diagnostic methods to identify the causes of MDR prior to the design and implementation of a therapeutic regimen.

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References

1. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58:** 137–171, 1989.
2. Chin K-V, Pastan I and Gottesman MM, Function and regulation of the human multidrug resistance gene. *Adv Cancer Res* **60:** 157–180, 1993.
3. Arceci RJ, Clinical significance of P-glycoprotein in multidrug resistance malignancies. *Blood* **81:** *2215–2222, 1993.*
4. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV and Deeley RG, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258:** 1650–1654, 1992.

5. Mirski SEL, Gerlach JH and Cole SPC, Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. *Cancer Res* **47:** 2594–2598, 1987.
6. Cole SPC, Chanda ER, Dicke FP, Gerlach JH and Mirski SEL, Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: Evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. *Cancer Res* **51:** 3345–3352, 1991.
7. Cole SPC, The 1991 Merck Frosst Award. Multidrug resistance in small cell lung cancer. *Can J Physiol Pharmacol* **70:** 313–329, 1992.
8. Higgins CF, ABC transporters: From microorganisms to man. *Annu Rev Cell Biol* **8:** 67–113, 1992.
9. Stride BD, Valdimarsson G, Gerlach JH, Cole SPC and Deeley RG, Structure and expression of the mRNA encoding the murine multidrug resistance protein (MRP), an ATP-binding cassette transporter. *Mol Pharmacol* **49:** 962–971, 1996.
10. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd AE, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J and Nelson DA, Cloning of the β cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science* **268:** 423–426, 1995.
11. Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, Borst P and Oude Elferink RPJ, Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271:** 1126–1128, 1996.
12. Szczypka MS, Wemmie JA, Moye-Rowley WS and Thiele DJ, A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. *J Biol Chem* **269:** 22853–22857, 1994.
13. Almquist KC, Loe DW, Hipfner DR, Mackie JE, Cole SPC and Deeley RG, Characterization of the 190 kDa multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. *Cancer Res* **55:** 102–110, 1995.
14. Ma L, Krishnamachary N and Center MS, Phosphorylation of the multidrug resistance associated protein gene encoded protein P190. *Biochemistry* **34:** 3338–3343, 1995.
15. Orr GA, Han EK-H, Browne PC, Nieves E, O'Connor BM, Yang C-PH and Horwitz SB, Identification of the major phosphorylation domain of murine *mdr*1b P-glycoprotein. Analysis of the protein kinase A and protein kinase C phosphorylation sites. *J Biol Chem* **268:** 25054–25062, 1993.
16. Chambers TC, Tohl J, Raynor RL and Kuo JF, Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268:** 4592–4595, 1993.
17. Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ and Smith AE, Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* **66:** 1027–1036, 1991.
18. Cole SPC, Patterns of cross-resistance in a multidrug-resistant small-cell lung carcinoma cell line. *Cancer Chemother Pharmacol* **26:** 250–256, 1990.
19. Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM and Deeley RG, Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **54:** 5902–5910, 1994.
20. Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA and Kruh GD, Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* **55:** 5342–5347, 1995.
21. Zaman GJR, Flens MJ, Van Leusden MR, de Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ and Borst P, The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci USA* **91:** 8822–8826, 1994.
22. Zaman GJR, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RPJ, Baas F and Borst P, Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc Natl Acad Sci USA* **92:** 7690–7694, 1995.
23. Loe DW, Deeley RG and Cole SPC, Biology of drug resistance associated with overexpression of the multidrug resistance protein, MRP. *Eur J Cancer* **32A:** 945–957, 1996.
24. Slapak CA, Mizunuma N and Kufe DW, Expression of the multidrug resistance associated protein and P-glycoprotein in doxorubicin-selected human myeloid leukemia cells. *Blood* **84:** 3113–3121, 1994.
25. Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deeley RG, Cole SPC and Sehested M, Sequential coexpression of the multidrug resistance genes *MRP* and *mdr1* and their products in VP-16 (etoposide)-selected H69 small cell lung cancer cells. *Cancer Res* **55:** 459–462, 1995.
26. Hasegawa S, Abe T, Naito S, Kotoh S, Kumazawa J, Hipfner DR, Deeley RG, Cole SPC and Kuwano M, Expression of multidrug resistance-associated protein (MRP), MDR1 and DNA topoisomerase II in human multidrug-resistant bladder cancer cell lines. *Br J Cancer* **71:** 907–913, 1995.
27. Reeve JG, Rabbitts PH and Twentyman PR, Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69. *Br J Cancer* **60:** 339–342, 1989.
28. Marsh W, Sicheri D and Center MS, Isolation and characterization of adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* **46:** 4053–4057, 1986.
29. Coley HM, Workman P and Twentyman PR, Retention of activity by selected anthracyclines in a multidrug resistant human large cell lung carcinoma line without P-glycoprotein hyperexpression. *Br J Cancer* **63:** 351–357, 1991.
30. Versantvoort CHM, Broxterman HJ, Pinedo HM, de Vries EGE, Feller N, Kuiper CM and Lankelma J, Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant lung cancer cell lines without P-glycoprotein expression. *Cancer Res* **52:** 17–23, 1992.
31. Schneider E, Horton JK, Yang C-H, Nakagawa M and Cowan KH, Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res* **54:** 152–158, 1994.
32. Flens MJ, Izquierdo MA, Scheffer GL, Fritz JM, Meijer CJLM, Scheper RJ and Zaman GJR, Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* **54:** 4557–4563, 1994.
33. Barrand MA, Robertson KJ, Neo S-Y, Rhodes T, Wright KA, Twentyman PR and Scheper RJ, Localisation of the multidrug resistance-associated protein, MRP, in resistant large-cell lung tumour cells. *Biochem Pharmacol* **50:** 1725–1729, 1995.
34. Marquardt D and Center MS, Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: Evidence for nuclear drug accumulation and redistribution in resistant cells. *Cancer Res* **52:** 3157–3163, 1992.
35. Krishnamachary N and Center MS, The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer Res* **53:** 3658–3661, 1993.
36. Flens MJ, Zaman GJR, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJLM and Scheper RJ, Tissue distribution of the multidrug resistance protein. *Am J Pathol* **148:** 1237–1247, 1996.
37. Cole SPC, Drug resistance and lung cancer. In: *Cancer: Concept to Clinic* (Ed. Wood J), pp. 15–21. Medical Publishing Enterprises, Fairlawn, NJ, 1992.

38. Coley HM, Amos WB, Twentyman PR and Workman P, Examination by laser scanning confocal fluorescence imaging microscopy of the subcellular localisation of anthracyclines in parent and multidrug resistant cell lines. *Br J Cancer* **67:** 1316–1323, 1993.
39. Sognier MA, Zhang Y, Eberle RL, Sweet KM, Altenberg GA and Belli JA, Sequestration of doxorubicin in vesicles in a multidrug-resistant cell line (LZ-100). *Biochem Pharmacol* **48:** 391–401, 1994.
40. Schuurhuis GJ, Broxterman HJ, de Lange JHM, Pinedo HM, van Heijningen THM, Kuiper CM, Scheffer GL, Scheper RJ, van Kalken CK, Baak JPA and Lankelma J, Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. *Br J Cancer* **64:** 857–861, 1991.
41. Huxham IM, Barlow AL, Lewis AD, Plumb J, Mairs RJ, Gaze MN and Workman P, Differential intracellular processing of the anthracycline drug ME2303 in doxorubicin-sensitive (A2780) and -resistant (A2780AD) human ovarian cancer cells as studied with confocal laser scanning microscopy and image analysis. *Int J Cancer* **59:** 94–102, 1994.
42. Molinari A, Cianfriglia M, Meschini S, Calcabrini A and Arancia G, P-glycoprotein expression in the Golgi apparatus of multidrug-resistant cells. *Int J Cancer* **59:** 789–795, 1994.
43. Gervasoni JE Jr, Fields SZ, Krishna S, Baker MA, Rosado M, Thuraisamy K, Hindenburg AA and Taub RN, Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy. *Cancer Res* **51:** 4955–4963, 1991.
44. Hindenburg AA, Gervasoni JE Jr, Krishna S, Stewart VJ, Rosado M, Lutzky J, Bhalla K, Baker MA and Taub RN, Intracellular distribution and pharmacokinetics of daunorubicin in anthracycline-sensitive and -resistant HL-60 cells. *Cancer Res* **49:** 4607–4614, 1989.
45. Rhodes T, Barrand MA and Twentyman PR, Modification by brefeldin A, bafilomycin A_1 and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) of cellular accumulation and intracellular distribution of anthracyclines in the non-P-glycoprotein-mediated multidrug-resistant cell line COR-L23/R. *Br J Cancer* **70:** 60–66, 1994.
46. Beck WT, The cell biology of multiple drug resistance. *Biochem Pharmacol* **36:** 2879–2887, 1987.
47. Sehested M, Skovsgaard T, Van Deurs B and Winther-Nielsen H, Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil. *Br J Cancer* **56:** 747–751, 1987.
48. Thiebaut F, Currier SJ, Whitaker J, Haugland RP, Gottesman MM, Pastan I and Willingham MC, Activity of the multidrug transporter results in alkalinization of the cytosol: Measurement of cytosolic pH by microinjection of a pH-sensitive dye. *J Histochem Cytochem* **38:** 685–690, 1990.
49. Keizer HG and Joenje H, Increased cytosolic pH in multidrug-resistant human lung tumor cells: Effect of verapamil. *J Natl Cancer Inst* **81:** 706–709, 1989.
50. Roepe PD, Wei LY, Cruz J and Carlson D, Lower electrical membrane potential and altered pH_i homeostasis in multidrug-resistant (MDR) cells: Further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein. *Biochemistry* **32:** 11042–11056, 1993.
51. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG and Keppler D, the *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* **269:** 27807–27810, 1994.
52. Muller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE and Jansen PLM, Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. *Proc Natl Acad Sci USA* **91:** 13033–13037, 1994.
53. Loe DW, Almquist KC, Deeley RG and Cole SPC, Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles: Demonstration of glutathione-dependent vincristine transport. *J Biol Chem* **271:** 9675–9682, 1996.
54. Hay DWP, Torphy TJ and Undem BJ, Cysteinyl leukotrienes in asthma: Old mediators up to new tricks. *Trends Pharmacol Sci* **16:** 304–309, 1995.
55. Jedlitschky G, Leier I, Buchholz U, Center M and Keppler D, ATP-dependent transport of glutathione *S*-conjugates by the multidrug resistance-associated protein. *Cancer Res* **54:** 4833–4836, 1994.
56. Hipfner DR, Gauldie SD, Deeley RG and Cole SPC, Detection of the M_r 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. *Cancer Res* **54:** 5788–5792, 1994.
57. Leier I, Jedlitschky G, Buchholz U, Center M, Cole SPC, Deeley RG and Keppler D, ATP-dependent glutathione disulfide transport mediated by the *MRP* gene-encoded conjugate export pump. *Biochem J* **314:** 433–437, 1996.
58. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G and Keppler D, Transport of glutathione, glucuronate, and sulfate conjugates by the *MRP* gene-encoded conjugate export pump. *Cancer Res* **56:** 988–994, 1996.
59. Loe DW, Almquist KC, Cole SPC and Deeley RG, ATP-dependent 17β-estradiol 17-(β-D-glucuronide) transport by multidrug resistance protein: Inhibition by cholestatic steroids. *J Biol Chem* **271:** 9683–9689, 1996.
60. Kitamura T, Jansen P, Hardenbrook C, Kamimoto Y, Gatmaitan Z and Arias IM, Defective ATP-dependent bile canalicular transport of organic anions in mutant (TR^-) rats with conjugated hyperbilirubinemia. *Proc Natl Acad Sci USA* **87:** 3557–3561, 1990.
61. Evers R, Zaman GJR, van Deemster L, Jansen H, Calafat J, Oomen LCJM, Oude Elferink RPJ, Borst P and Schinkel AH, Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *J Clin Invest* **97:** 1211–1218, 1996.
62. McGrath T, Latoud C, Arnold ST, Safa AR, Felsted RL and Center MS, Mechanisms of multidrug resistance in HL60 cells: Analysis of resistance associated membrane proteins and levels of *mdr* gene expression. *Biochem Pharmacol* **38:** 3611–3619, 1989.
63. Versantvoort CHM, Broxterman HJ, Bagrij T, Scheper RJ and Twentyman PR, Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. *Br J Cancer* **72:** 82–89, 1995.
64. Neyfakh AA, Use of fluorescent dyes as molecular probes for the study of multidrug resistance. *Exp Cell Res* **174:** 168–176, 1988.
65. Homolya L, Hollo Z, Germann UA, Pastan I, Gottesman MM and Sarkadi B, Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J Biol Chem* **268:** 21493–21496, 1993.
66. Weaver JL, Pine PS, Aszalos A, Schoenlein PV, Currier SJ, Padmanabhan R and Gottesman MM, Laser scanning and confocal microscopy of daunorubicin, doxorubicin, and rhodamine 123 in multidrug-resistant cells. *Exp Cell Res* **196,** 323–329, 1991.
67. Lahmy S, Lautier D, Canitrot Y, Laurent G and Salmon J-M, Staining with Hoechst 33342 and rhodamine 123: An attempt to detect multidrug resistant phenotype cells in leukemia. *Leuk Res* **17:** 1021–1029, 1993.
68. Canitrot Y, Lautier D, Lahmy S, Vigo J, Viallet P and Salmon J-M, Nile red labeling of single living cells for contour delineation to quantify and evaluate the distribution of rhodamine

123 with fluorescence image cytometry. *J Histochem Cytochem* **41:** 1785–1793, 1993.
69. Lautier D, Canitrot Y and Salmon J-M, Effects of vinblastine, colchicine, and verapamil on rhodamine 123 accumulation in human P-glycoprotein-positive leukemia cells. *Anticancer Res* **14:** 2589–2596, 1994.
70. Canitrot Y, Lautier D, Viallet P and Salmon J-M, Modulation of rhodamine 123 uptake by nigericin in sensitive and multidrug resistant leukemic cells. *Cancer Lett* **84:** 75–83, 1994.
71. Twentyman PR, Rhodes T and Rayner S, A comparison of rhodamine 123 accumulation and efflux in cells with P-glycoprotein-mediated and MRP-associated multidrug resistance phenotypes. *Eur J Cancer* **30A:** 1360–1369, 1994.
72. Feller N, Kuiper CM, Lankelma J, Ruhdal JK, Scheper RJ, Pinedo HM and Broxterman HJ, Functional detection of *MDR1*/P170 and *MRP*/P190 mediated multidrug resistance in tumor cells by flow cytometry. *Br J Cancer* **72:** 543–549, 1995.
73. Feller N, Broxterman HJ, Wahrer DCR and Pinedo HM, ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): No inhibition by intracellular glutathione depletion. *FEBS Lett* **368:** 385–388, 1995.
74. Versantvoort CHM, Bagrij T, Wight KA and Twentyman PR, On the relationship between the probenecid-sensitive transport of daunorubicin or calcein and the glutathione status of cells overexpressing the multidrug resistance-associated protein (MRP). *Int J Cancer* **63:** 855–862, 1995.
75. Versantvoort CHM, Broxterman HJ, Lankelma J, Feller N and Pinedo HM, Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact *MRP* overexpressing human small cell lung cancer cells. *Biochem Pharmacol* **48:** 1129–1136, 1994.
76. Versantvoort CHM, Schuurhuis GJ, Pinedo HM, Eekman CA, Kuiper CM, Lankelma J and Broxterman H, Genistein modulates the decreased drug accumulation in non-P-glycoprotein mediated multidrug resistant tumour cells. *Br J Cancer* **68:** 939–946, 1993.
77. Nooter K, Westerman AM, Flens MJ, Zaman GJR, Scheper RJ, van Wingerden KE, Burger H, Oostrum R, Boersma T, Sonneveld P, Gratama JW, Kok T, Eggermont AMM, Bosman FT and Stoter G, Expression of the multidrug resistance-associated protein (*MRP*) gene in human cancers. *Clin Cancer Res* **1:** 1301–1310, 1995.
78. Kruh GD, Gaughan KT, Godwin A and Chan A, Expression pattern of MRP in human tissues and adult solid tumor cell lines. *J Natl Cancer Inst* **87:** 1256–1258, 1995.
79. Ford FM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155–159, 1991.
80. Cole SPC, Downes HF and Slovak ML, Effect of calcium antagonists on the chemosensitivity of two multidrug resistant human tumour cell lines which do not overexpress P-glycoprotein. *Br J Cancer* **59:** 42–46, 1989.
81. Barrand MA, Rhodes T, Center MS and Twentyman PR, Chemosensitisation and drug accumulation effects of cyclosporin A, PSC-833 and verapamil in human MDR large cell lung cancer cells expressing a 190k membrane protein distinct from P-glycoprotein. *Eur J Cancer* **29A:** 408–415, 1993.
82. Gollapudi S and Gupta S, Lack of reversal of daunorubicin resistance in HL60/AR cells by cyclosporin A. *Anticancer Res* **12:** 2127–2132, 1992.
83. Abe T, Koike K, Ohga T, Kubo T, Wada M, Kohno K, Mori T, Hidaka K and Kuwano M, Chemosensitization of spontaneous multidrug resistance by a 1,4-dihydropyridine analog and verapamil in human glioma cell lines overexpressing MRP or MDR1. *Br J Cancer* **72:** 418–423, 1995.
84. Gollapudi S, Thadepalli F, Kim CH and Gupta S, Difloxacin reverses multidrug resistance in HL-60/AR cells that overexpress the multidrug resistance-related protein (MRP) gene. *Oncol Res* **7:** 213–225, 1995.
85. Gekeler V, Boer R, Ise W, Sanders KH, Schachtele C and Beck J, The specific bisindolylmaleimide PKC-inhibitor GF 109203X efficiently modulates MRP-associated multiple drug resistance. *Biochem Biophys Res Commun* **206:** 119–126, 1995.
86. Gekeler V, Ise W, Sanders KH, Ulrich W-R and Beck J, The leukotriene LTD_4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* **208:** 345–352, 1995.
87. Cole SPC, Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemother Pharmacol* **17:** 259–263, 1986.
88. Cole SPC, Downes HF, Mirski SEL and Clements DJ, Alterations in glutathione and glutathione-related enzymes in a multidrug resistant small cell lung cell line. *Mol Pharmacol* **37:** 192–197, 1990.
89. Schneider E, Yamazaki H, Sinha BK and Cowan KH, Buthionine sulphoxime-mediate sensitisation of etoposide-resistant human breast cancer MCF7 cells overexpressing the multidrug resistance-associated protein involves increased drug accumulation. *Br J Cancer* **71:** 738–743, 1995.
90. Tietze F, Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal Biochem* **27:** 502–522, 1969.
91. Davey RA, Longhurst TJ, Davey MW, Belov L, Harvie RM, Hancox D and Wheeler H, Drug resistance mechanisms and MRP expression in response to epirubicin treatment in a human leukaemia cell line. *Leuk Res* **19:** 275–282, 1995.
92. Lutzky J, Astor MB, Taub RN, Baker MA, Bhalla K, Gervasoni JE Jr, Rosado M, Stewart V, Krishna S and Hindenburg AA, Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells. *Cancer Res* **49:** 4120–4125, 1989.
93. Ford JM, Yang J-m and Hait WN, Effect of buthionine sulfoximine on toxicity of verapamil and doxorubicin to multidrug resistant cells and to mice. *Cancer Res* **51:** 67–72, 1991.
94. Holm PS, Dietel M and Krupp G, Similar cleavage efficiencies of an oligoribonucleotide substrate and an *mdr1* mRNA segment by a hammerhead ribozyme. *Gene* **167:** 221–225, 1995.
95. Kiehntopf M, Brach MA, Licht T, Petschauer S, Karawajew L, Kirschning C and Herrmann F, Ribozyme-mediated cleavage of the *MDR-1* transcript restores chemosensitivity in previously resistant cancer cells. *EMBO J* **13:** 4645–4652, 1994.
96. Quattrone A, Papucci L, Morganti M, Coronnello M, Mini E, Mazzei T, Colonna FP, Garbesi A and Capaccioli S, Inhibition of *MDR1* gene expression by antimessenger oligonucleotides lowers multiple drug resistance. *Oncol Res* **6:** 311–320, 1994.
97. Stewart AJ, Canitrot Y, Baracchini E, Dean NM, Deeley RG and Cole SPC, Reduction in the expression of the multidrug resistance protein, MRP, in human tumour cells by antisense phosphorothioate oligonucleotides. *Biochem Pharmacol* **51:** 461–469, 1996.