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Experimental Modulation of MRP (multidrug resistance-associated protein)-mediated Resistance

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INTRODUCTION

DURING THE years following the experimental description of the phenomenon of "multidrug resistance", its association with decreased cellular accumulation of the involved drug, and the identification of P-glycoprotein as the underlying mechanism, many laboratories around the world began to isolate their own multidrug resistance (MDR) cell lines. For reasons which are still not entirely clear (to us at least), most investigators used doxorubicin (Adriamycin) as the selecting agent, although the cytotoxic action of this agent is complex and probably involves multiple mechanisms. Several laboratories working independently, but in parallel, succeeded in isolating MDR cell lines, which shared many features with lines having a "classical MDR" mechanism (that is, involving P-glycoprotein), but in which overexpression of neither P-glycoprotein nor of mRNA from the encoding *MDR1* gene could be detected. A line isolated by Danks and colleagues by growth of cells in teniposide (VM26), whilst crossresistant to etoposide and the anthracyclines, showed no crossresistance to the vinca alkaloids and did not have a drug accumulation deficit [1]. The authors referred to this phenotype as "atypical MDR" and subsequently demonstrated that abnormalities in function of the nuclear enzyme, topoisomerase II, were responsible [2]. However, many of the other lines showed a clear deficit in drug accumulation and a full spectrum of cross-resistance. In the absence of any clues to the mechanism, this phenotype became referred to as "non-P-glycoprotein-mediated MDR". It was not, of course, known whether all such lines (Table 1) [3–9] shared a common mechanism or whether a number of alternative routes to MDR existed.

Table 1. Early non-P-glycoprotein mediated MDR cell lines

Line	Type	[Ref.]
H69AR	Small cell lung carcinoma	[3]
COR-L23/R	Large cell lung carcinoma	[4]
MOR/R	Lung adenocarcinoma	[4]
GLC_4/ADR	Small cell lung carcinoma	[5]
SW-1573/2R120	Non-small cell lung carcinoma	[6]
HL60/Adr	Leukaemia	[7]
HL60/ADR	Leukaemia	[8]
HT1080/DR4	Fibrosarcoma	[9]

In 1989, the first clues to the mechanism came from the work of Center and colleagues [10]. They derived a series of 15 synthetic peptides from the deduced amino acid sequence of P-glycoprotein. Polyclonal rabbit antisera were then raised to each of the peptides and used to probe on immunoblots membranes prepared from both a P-glycoprotein-expressing MDR cell line and also a human leukaemia MDR line (HL60/Adr) in which P-glycoprotein could not be detected. Each of the antisera recognised a 170 kD band in the P-glycoprotein positive cells but, in addition, one also recognised a band of around 190 kD in the HL60/Adr cells. The antiserum in question (ASP14) had been raised against a 15 amino acid sequence overlapping the c-terminal ATP-binding site of P-glycoprotein, a region known to be highly conserved in such ATP-binding cassette-transport proteins. We obtained a sample of ASP14 antiserum and found that a 190 kD protein was also overexpressed in our COR-L23/R lung cancer MDR cell line. Subsequent studies, in collaboration with Drs Broxterman and Versantvoort in The Netherlands, using a similar antiserum, derived at the MRC Clinical Oncology and Radiotherapeutics Unit, also found a protein of similar size in three other independently derived non-P-glycoprotein MDR lung cancer cell lines [11]. It was clear that an alternative route to an MDR phenotype involved overexpression of a transporter protein, which shared some sequence with P-glycoprotein, but which was somewhat larger in size. Several groups began attempts to clone the gene coding for the 190 kD protein using a variety of strategies and the group to win the race was that of Cole and Deeley in Canada who, in December 1992, published the cDNA sequence of a gene on chromosome 16 that coded for a protein which they called MRP (for Multidrug Resistance-associated Protein) [12]. We and others were able to show that the MRP protein was indeed the 190 kD protein expressed in many non-P-glycoprotein-mediated MDR cell lines [13–15]. Subsequently, gene transfection studies confirmed that expression of the *MRP* gene was itself able to confer an MDR phenotype to the resistant cells [16, 17].

In 1994, a group in Germany, followed shortly afterwards by a Dutch group, published data indicating that MRP was almost certainly identical to the glutathione *S*-conjugate transporter present in a variety of normal cell types [18, 19]. Natural substrates for the transporter include leukotriene LTC_4 and reduced glutathione GSH. These observations,

moreover, have recently provided an exciting new area of investigation with respect to the biology of MRP and strategies for its circumvention.

As soon as the non-P-glycoprotein-mediated MDR phenotype was identified as a separate entity, studies of possible routes to resistance reversal began. Naturally, the first compounds to be studied were those which had already been shown to be effective modulators of resistance mediated by P-glycoprotein. Further groups of compounds, selected on the basis of a relationship between their properties and the biology of non-P-glycoprotein-mediated resistance, have also been studied. Following the identification of MRP as the mechanism responsible and its identification as a glutathione *S*-conjugate transporter, a third area of approach has been opened. These three stages towards our current understanding of the possibility of reversal of MRP-mediated MDR form the basis of this review.

PGP MODULATORS

Our understanding of many aspects of the drug resistance profile, resulting from overexpression of P-glycoprotein in mammalian cells, remains incomplete. The relative resistance factors for different agents varies from cell line to cell line, and there is a tendency (but not a very strong one) for resistance to the selecting agent to be greater than that to other agents within the phenotype. It was originally thought that this may be due to the presence of multiple genes coding for closely related proteins, but it is now clear that only a single gene codes for a drug-resistance conferring P-glycoprotein in humans compared with two genes in the mouse. It is of course possible that isolation of MDR sublines by drug selection produces a variety of additional mechanistic changes. Although overexpression of P-glycoprotein may be the predominant mechanism, the precise resistance phenotype may also be determined by the co-existence of other mechanisms. Alternative explanations for the variation in phenotype involve conformational differences in P-glycoprotein, resulting in changes in relative accessibility or activity of different drug-binding sites, differential dependence of different drugs on post-translational modification (for example, phosphorylation) of P-glycoprotein and differences in cellular pharmacodynamic handling (e.g. plasma membrane residence time) between different drugs. These same uncertainties become greater when combinations of cytotoxic drugs and resistance modulators have to be considered. There is no doubt, based on published data from laboratory studies using MDR cell lines, that different modulators have different relative efficacies when combined with different cytotoxics. The following discussion of modulators of P-glycoprotein-mediated MDR is, therefore, necessarily somewhat general in that it does not emphasise the variabilities seen between cell lines and between cytotoxics. However, such differences tend to be somewhat subtle in comparison with the most important and generalised observations.

In attempting to find modulators of multiple drug resistance, where membrane changes were clearly involved, it seemed logical to examine a range of membrane-active compounds. This was the approach taken by Professor Tsuruo in Tokyo which led to his description in 1981 of the calcium channel blocker, verapamil, and the calmodulin inhibitor, trifluoperazine as specific modulators of MDR in a mouse leukemia cell line [20]. It was originally thought that the effectiveness of these agents may be related to their effects on calcium channels, but the maintenance of verapamil chemosensitisation in MDR cells lacking voltage-dependent calcium channels disproved this hypothesis. A large number of other calcium antagonists were subsequently examined and compared with verapamil for effectiveness as MDR modulators. It was not possible in patients to achieve plasma levels of these early compounds, which could be predicted from *in vitro* studies to produce adequate reversal of MDR. Many groups, therefore, began to examine alternative compounds. Our group in Cambridge began to investigate the chemosensitisation properties of the immunosuppressive cyclic peptide, cyclosporin A. Unknown to us, a similar line of investigation was being pursued by Dr Lew Slater in California and his publication of cyclosporin A as an effective modifier of P-glycoprotein-mediated MDR preceded ours by some months [21, 22]. Because of its potent immunosuppression and also because, once again, it appeared that adequate plasma levels could not be achieved in patients, cyclosporin A still left much to be desired as a clinical modifier. We therefore examined a series of non-immunosuppressive analogues supplied by Sandoz and found that several were more potent as resistance modifiers than the parent compound [23]. Further work at Sandoz resulted in the identification of an analogue, SDZ PSC-833, which was non-immunosuppressive and 10–20-fold more potent than cyclosporin A [24]. This has become the first second-generation modifier to reach clinical trials, developed specifically for this purpose. It has completed a number of Phase I trials and is now in larger Phase II and III trials around the world. Other compounds that have also been extensively studied as modifiers of P-glycoprotein-mediated MDR include quinine and quinidine, the anti-oestrogen, tamoxifen, dexniguldipine, and the acridone carbocamide derivative, GF120918 (reviewed in [25]). These compounds have generally been shown to restore the defective drug accumulation seen in P-glycoprotein-mediated MDR cells and to displace binding of the photoactive calcium antagonist, azidopine, from a 170 kD band on protein gels prepared from membranes of MDR cells. It is, therefore, assumed that their primary mode of action is competition for drug-binding sites on the P-glycoprotein molecule. Indeed, photoactive analogues of verapamil and cyclosporin A have been shown to bind directly to the 170 kD band [26, 27].

Several authors used their own non-P-glycoprotein-mediated MDR cell lines to examine the effects of modulators. The H69AR lung cancer line, from which the *MRP* gene was isolated, is unique amongst MRP-overexpressing lines in that it does not have a drug accumulation deficit. It has been suggested that, in this cell line, MRP is expressed mainly on internal membranes resulting in changes in intracellular drug accumulation rather than gross cellular drug content. By contrast, the HT1080/DR4 cell line has a clear accumulation deficit. A 1989 study of these two cell lines revealed that both verapamil and cyclosporin A had only a modest effect as reversers of resistance and that verapamil was the more effective of the two [28]. We reached the same conclusions in studies carried out using our MRP-overexpressing lung cancer cell lines, COR-L23/R and MOR/R. Furthermore, we found that the cyclosporin analogue, SDZ PSC-833, used at a concentration of 2 μM, had little if any effect in contrast to its usual 10-fold advantage over cyclosporin A in most P-glycoprotein-mediated cell lines studied [29] (Figure 1). Two additional studies also lead to the same general conclusions [30, 31]. One publication, however, contains contradictory

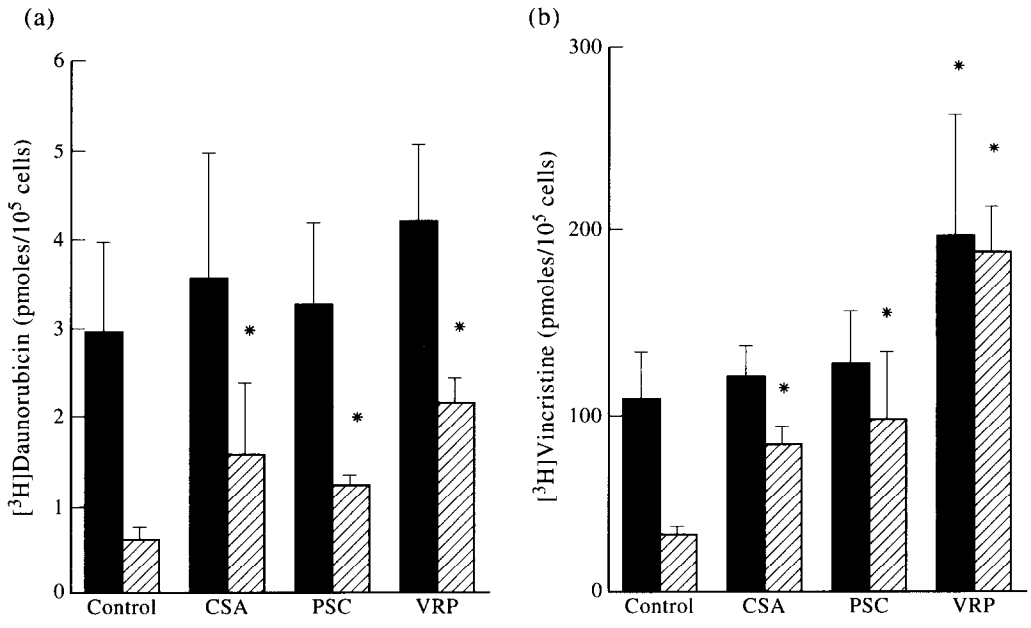

Figure 1. Accumulation of (a) [^{3}H]daunorubicin and of (b) [^{3}H]vincristine into L23/P (solid bars) and L23/R cells (hatched bars) following exposure to drug alone (control), or to drug in the presence of 5 μg/ml cyclosporin A (CSA), 5 μg/ml PSC-833 (PSC) or 3.3 μg/ml verapamil (VRP). The values shown are the mean of data derived from three separate experiments, triplicate determinations being obtained from each; bars, S.D. *Statistically different ($P < 0.01$) compared to control (Student's t-test). Reproduced from Barrand MA, *et al.*, *Eur J Cancer* 1993, Vol. 29A, pp. 408–415.

results [32], i.e. in the MRP-overexpressing cell line HL60/Adr, the effects of cyclosporin A and PSC-833 upon cellular accumulation of daunorubicin, determined by flow cytometry, led to essentially complete reversal of the deficit. In an MRP-transfected Hela cell line, the effects of verapamil and cyclosporin A upon sensitivity to doxorubicin or vincristine were little different to those in vector-only transfected controls [33]. The reason for this discrepancy is unknown at least partly because, in contrast to the situation for P-glycoprotein, there is no good evidence for binding of either cytotoxic drugs or of resistance modifiers to the MRP protein. Hence, the mechanism by which typical P-glycoprotein modulators can, in some cell lines at least, reverse resistance mediated by MRP and reverse the associated drug accumulation deficit is unclear.

NOVEL APPROACHES

Several groups have reported striking differences in the intracellular distribution of anthracyclines between parental cells and their MRP-overexpressing variants. Whereas intracellular fluorescence was mainly in the nucleus of parental COR-L23 lung cancer cells, the brightest fluorescence in the COR-L23/R resistant cells was confined to groups of perinuclear vesicles with a "Golgi-like" distribution [34]. In the HL60/Adr leukaemia MRP-overexpressing subline, Marquardt and Center found that daunorubicin initially entered the nucleus of the cells, but was subsequently redistributed into cytoplasmic vesicles [35]. In these cells, MRP was expressed at high levels on the endoplasmic reticulum. The authors proposed a pathway of drug extrusion that involved the concentration of drugs into cytoplasmic vesicles followed by an endocytotic process transporting the drug to the exterior of the cell. As an approach to inhibiting this pathway, they studied the effects of vacuolar H+ ATPase inhibitors, bafilomycin A1 and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD), on drug accumulation and intracellular distribution [36]. They also studied the effects of the fungal antibiotic, brefeldin A, which had been identified as an inhibitor of protein recycling from the endoplasmic reticulum into the Golgi body [34]. Bafilomycin A1 and NBD had no effect on accumulation of daunorubicin in the parental cells, but caused a clear increase in both HL60/Adr and in a P-glycoprotein overexpressing subline HL60/Vinc. In contrast, no clear differential effect was seen with brefeldin A. Unfortunately, no data were presented from which the toxicity of the three compounds at the concentrations used could be determined. We therefore subsequently examined the effects of the same three compounds in our COR-L23/R cells, with particular attention paid to the relationship between effects on drug accumulation and toxicity at different concentrations [37]. We found that all three agents could produce selective reversal of the resistance-related accumulation defect for daunorubicin, but that this was only seen at concentrations which were themselves equal to or greater than those which alone produced clear effects on cell growth. In a direct chemosensitivity assay, none of the agents, at maximum non-toxic concentrations, was able to sensitise COR-L23/R cells to doxorubicin or to colchicine. It seemed unlikely, therefore, that such an approach would prove useful in the development of clinical sensitisers.

Various studies had shown that the activity of P-glycoprotein could be modulated by phosphorylation and that various protein kinase inhibitors could reduce the level of resistance in P-glycoprotein overexpressing cells [38]. In a cell line in which resistance was not due to P-glycoprotein, the tyrosine kinase inhibitor, genistein, was also shown to modulate intracellular distribution of doxorubicin [39]. The group of Dr Broxterman, therefore, studied the effect of both staurosporine (an inhibitor of protein kinase C) and genistein in a panel of parental, P-glycoprotein overexpressing and MRP-overexpressing cell lines [40]. Large effects of staurosporine were seen in the P-glyco-

protein MDR cells, but the effects were minimal in cells overexpressing MRP. In contrast, genistein had little effect on P-glycoprotein mediated resistance, but it increased accumulation and decreased efflux of daunorubicin in cells overexpressing MRP. Three other (iso)flavonoids, biochanin A, apigenin and quercetin, were also effective despite having minimal activity as tyrosine kinase inhibitors. Similar to the modulation of Pgp, the mechanism involved appears to be competition for drug binding sites. Unfortunately, the effect of genistein was again only seen at concentrations that were themselves too toxic for longer-term cell exposure. It is unlikely, therefore, to be clinically useful as a resistance reversal agent. Nevertheless, modulation of daunorubicin accumulation by genistein has been proposed as a specific functional test for MRP activity in clinical leukaemia specimens.

As the fluorescent compound, rhodamine 123, was becoming widely used as a functional probe for P-glycoprotein activity, particularly in haemopoietic cells, we decided to examine its handling by cells overexpressing MRP. We found that, in our COR-L23/R cells, there was reduced accumulation and increased efflux of rhodamine 123 compared with the parental line [41]. However, the differential was only seen over a longer time period than in P-glycoprotein overexpressing cells. We confirmed the effect of genistein upon daunorubicin accumulation (i.e. reversal of the accumulation deficit) in our COR-L23/R cells and examined the interaction between genistein and rhodamine 123. Surprisingly, the effect was in the opposite direction (i.e. genistein increased the effect of MRP activity and resulted in a greater accumulation deficit) [42]. Two other (iso)flavanoids, diadzein and quercetin, also accelerated rhodamine efflux from the resistant line whilst genistin (which differs from genistein only by the addition of a glucose molecule) did not. It is clear from these data that closely related flavonoids can have quite different effects on daunorubicin transport in MRP-overexpressing cells and that effects on different fluorescent MRP substrates can be in opposite directions. These differences may relate to the different intracellular compartmentalisation of different substrates and hence their accessability for MRP action.

Most of the well-characterised non-Pgp MDR cells have been selected for resistance to doxorubicin [3–9]. This may induce a variety of resistance mechanisms including increased drug efflux via (i) overexpression of Pgp or MRP; (ii) reduced DNA topoisomerase II activity; or (iii) more effective detoxification of doxorubicin and/or doxorubicin-induced radicals by glutathione (GSH) [43, 44]. Elevated levels of GSH, together with increased activities of glutathione *S*-transferase (GST) or peroxidase, may protect cells from cytotoxic drugs such as melphalan, platinum compounds and anthracyclines (reviewed in [44]). Consequently, depletion of cellular GSH by exposure to buthionine sulphoximine (BSO), a potent inhibitor of GSH synthesis, can result in increased cytotoxicity of doxorubicin in Pgp–MDR tumour cell lines [45, 46]. In Pgp–MDR MCF7/ADR cells, decreased accumulation of doxorubicin was not affected by BSO treatment, but an increase in formation of free radicals was seen. Hence, an increased detoxification by the GSH/GST system was partly responsible for the resistance to doxorubicin [46].

In early studies on non-P-glycoprotein-mediated MDR, BSO was found to increase doxorubicin/daunorubicin (DNR) toxicity in several cell lines [47–49]. In the GLC4/ADR cells used in one of these studies, GSH and GST levels were increased two-fold compared to the parental cells, and it was, therefore, suggested that detoxification of doxorubicin by GSH/GST system was an important factor contributing to doxorubicin resistance [48]. However, in HL60/AR cells, GSH and GST levels were decreased compared to the parental cells and potentiation of DNR toxicity was accompanied by an increase in cellular DNR accumulation [47]. Although in the H69/AR cell line the toxicity of doxorubicin was not potentiated by BSO [50], only 1 μM BSO was used in that study (because of the sensitivity of the resistant cells to BSO), whereas in the other studies 25–50 μM BSO was used. GLC4/ADR, HL60/AR and H69/AR cells have subsequently each been found to overexpress the *MRP* gene [12–14]. Hence, although BSO is able to reverse resistance to anthracyclines in some Pgp- and MRP-mediated MDR cells, the mechanism is unclear, and whether or not BSO could reverse resistance to other cytotoxic agents involved in the MDR phenotype had not been investigated.

In a recently published study [51], we investigated whether or not BSO is a more general resistance modifier for MRP-mediated MDR. Effects of BSO treatment on the sensitivity of cells to daunorubicin, vincristine and rhodamine 123 were studied in three MRP-overexpressing, MDR human lung tumour cell lines, COR-L23/R, MOR/R and GLC4/ADR. In addition, we examined the effects of BSO on daunorubicin and rhodamine 123 transport and related these changes to cellular GSH levels. Resistance to daunorubicin, vincristine and rhodamine 123 was partially reversed in these cell lines following exposure of the cells to BSO. This was associated with an increased intracellular accumulation of daunorubicin and rhodamine 123, due to inhibition of enhanced drug efflux (Figure 2). In contrast, in a P-glycoprotein-mediated MDR cell line, the accumulation of daunorubicin was not increased by BSO treatment. BSO treatment (25 μM, 20 h) of the cell lines resulted in 60–80% depletion of cellular GSH levels. Changes in daunorubicin accumulation in COR-L23/R and GLC4/ADR cells were closely associated with cellular GSH depletion by BSO treatment. Furthermore, increase in cellular GSH levels in BSO-treated COR-L23/R and GLC4/ADR cells by incubation with 5 mM GSH-ethylester led to restoration of the daunorubicin accumulation deficit. However, transport of daunorubicin did not increase the GSH release in any of the cell lines. These results demonstrate that drug transport in MRP but not in Pgp-overexpressing MDR tumour cell lines can be regulated by intracellular GSH levels. However, the expression of MRP protein was not affected by GSH depletion, implying an effect based on protein function. We concluded, therefore, that drug transport is regulated differently in MRP- and Pgp-overexpressing cells [51].

ORGANIC ANION TRANSPORT INHIBITORS

Recently, it has been shown that MRP is identical to the GSH *S*-conjugate transporter present in a variety of normal cell types [18, 19]. Natural substrates for this transport system include leukotriene LTC_4, *s*-dinitrophenylglutathione, and oxidised glutathione (GSSG) each of which are organic anions. Consequently, a logical approach to modulation of MRP activity involved the use of organic ion transport inhibitors. Using, as a model system, the transport of LTC_4 into membrane vesicles prepared from mastocytoma cells, Leier and colleagues showed effective inhibition by both probenecid (IC_{50} = 71 μM) and the LTD_4 receptor antagonist, MK571 (IC_{50} = 1 μM) [52]. The same group then confirmed these results using membrane vesicles from MRP-transfected Hela cells obtaining K_i values of 0.6 μM for MK571, 5 μM for

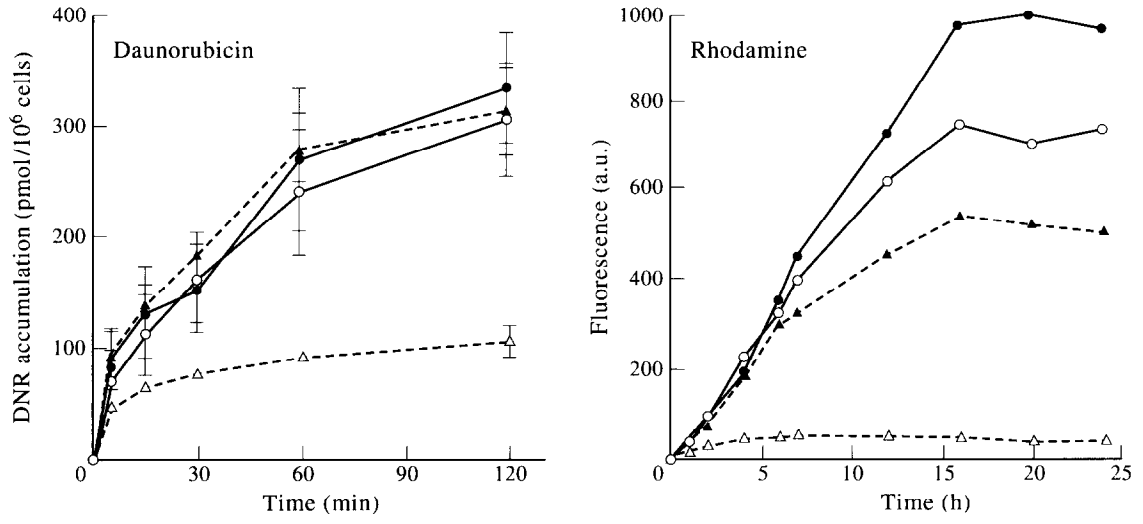

Figure 2. Effect of BSO on DNR and Rh 123 uptake in COR-L23 cells. COR-L23/P (circles) and COR-L23/R (triangles) were incubated in presence of 25 μM BSO for 20–28 h (closed symbols) or vehicle (open symbols). Cellular accumulation of 0.5 μM ^{3}H-DNR (data are mean ± S.D. from three experiments, each performed in triplicate) and 0.01 μg/ml Rh 123 (a representative experiment out of three experiments is shown) was determined at the time points indicated. Reproduced with permission from Versantvoort CHM, *et al., Br J Cancer* 1995, Vol. 72, pp. 82–89.

cyclosporin A and 27 μM for PSC-833 [53]. The Dutch group again used a very similar system with membrane vesicles from their own MRP-transfected cell line and examined the effects of various compounds on ATP-dependent transport of radiolabelled LTC_4 [19]. They showed inhibition by the alternative substrates, GSSG and Nonyl-GS, as well as the ATPase inhibitor, sodium orthovanadate. Inhibition by vinca alkaloids (but not doxorubicin) was also seen (Table 2). In MRP-overexpressing whole cell systems, Gekeler and colleagues showed that MK571 could reverse completely vincristine resistance whilst having no effect in P-glycoprotein overexpressing cells [54]. The same group were also able to show complete or partial reversal of MRP-mediated cellular resistance by the highly selective bisindolylmaleimide protein kinase C inhibitor, GF 109203X [55].

Sarkadi and associates have described the use of calcein acetoxymethyl ester (calcein AM) as a particularly efficient functional probe for Pgp activity [56, 57]. Calcein AM is a non-fluorescent substrate which is cleaved by cellular esterases to produce the fluorescent derivative, calcein. Calcein is an organic anion as are GSH *S*-conjugates and hence a possible substrate for MRP-mediated transport. We therefore characterised the transport of calcein in cells that overexpress the MRP transporter and examined how such transport is modified following GSH depletion by BSO. In addition, we examined the effect of various chemical modulators on daunorubicin or calcein accumulation/efflux in relation to cellular GSH transport [58]. Accumulation of calcein fluorescence was greatly reduced in the MRP-overexpressing human lung cancer cell lines, COR-L23/R and MOR/R, compared with their parental lines. Energy depletion resulted in a considerably increased accumulation in the resistant lines. Reduced accumulation of calcein in MRP-overexpressing cells was also independently described by Feller and colleagues [32]. Treatment of resistant cells with BSO did not affect calcein accumulation, in marked contrast to our previous results for daunorubicin or the fluorescent probe, rhodamine 123. Again, results from Dr Broxterman's laboratory led them to a similar

*Table 2. Effect of inhibitors on ATP-dependent [^{3}H]LTC_4 transport into plasma membrane vesicles from S1(MRP) cells**

Treatment		
Drug	Conc. (μM)	[^{3}H]LTC_4 uptake (%)
None	—	100
GSSG	100	75 ± 6†
	500	48 ± 2†
Nonyl-GS	10	14 ± 2†
	100	1 ± 1†
GSH	5000	106 ± 3
Ouabain	1000	100 ± 7
Doxorubicin	100	100 ± 10
Vincristine	10	90 ± 1
	100	52 ± 3†
Vinblastine	10	91 ± 2
	100	36 ± 3†
Vanadate	50	60 ± 2†

*Plasma membrane-enriched vesicles from the stable *MRP* transfectant S1(MRP) were incubated with [^{3}H]LTC_4 (1.25 nM) in the presence or absence (control) of the indicated compounds. Relative transport rates (per cent of control) were calculated by subtracting the values in the presence of ATP from those in the presence of 5'-AMP (means ± S.D. from at least two experiments, each performed in triplicate.

†Bonferroni P value < 0.001 (ANOVA).

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conclusion [59]. We found that genistein, verapamil, cyclosporin A and ouabain were each able to modify, to some extent, accumulation of daunorubicin, whilst having essentially no effect on calcein accumulation [58]. However, the organic anion transport inhibitor, probenecid, was able to increase accumulation of both calcein and daunorubicin in the resistant cells (Figure 3). Genistein and verapamil treatment preferentially reduced the GSH content of resistant cells, whilst probenecid did not. However, probenecid caused a clear decrease in release of GSH from resistant cells into the medium. We believe that calcein acts as a substrate for the MRP pump in the same way as do GSH *S*-conjugates. Transport of these organic anions is sensitive to probenecid and is necessary, but not sufficient, for transport of daunorubicin and rhodamine 123. Further processes are involved in transport of these latter compounds and these processes are sensitive to a range of other modulators.

It appears likely that calcein is effluxed from MRP-overexpressing cells via a markedly different pathway from that which transports cytotoxic drugs, such as daunorubicin. Calcein transport may be analogous to that of leukotriene LTC_4. We have speculated previously that the activity of the MRP transporter may, in some way, regulate a related mechanism that is responsible for daunorubicin transport [51]. Our new data for probenecid support such an idea. It appears likely that probenecid inactivates the MRP pump for which GSH *S*-conjugates (including LTC_4) or calcein act as substrates. Efflux of DNR and rhodamine 123 occurs via a mechanism for which MRP action is necessary, but not sufficient. Our data indicate that supplying the MRP pump with a high concentration of calcein does not modulate its ability to efflux DNR, and hence direct competition appears unlikely. The additional function required for efflux of DNR and rhodamine 123 is sensitive to a number of additional inhibitors including genistein, verapamil and ouabain. These may act in different ways, possibly by direct competition [60] by inhibition of ATPase activity (ouabain) or cellular GSH levels. The effects of genistein and verapamil on cellular GSH and GSH release strongly suggest an involvement of GSH biochemistry in drug transport.

Although MRP-overexpressing cells show a greatly reduced accumulation of calcein compared with their parental counterparts, the use of calcein AM as a functional probe must be currently regarded with caution. Effects of modifers on calcein accumulation clearly do not predict for effects on daunorubicin accumulation. Furthermore, as calcein is effluxed from COR-L23/R cells (i.e. MRP-overexpressing), but not from H69/LX4 cells (Pgp-overexpressing) [58], the kinetics underlying reduced accumulation are clearly different in the two types of resistance. Further studies involving a wider range of substrates and modifiers together with a more detailed examination of GSH kinetics may help to elucidate the nature of the distinct transport processes associated with MRP overexpression (Figure 4).

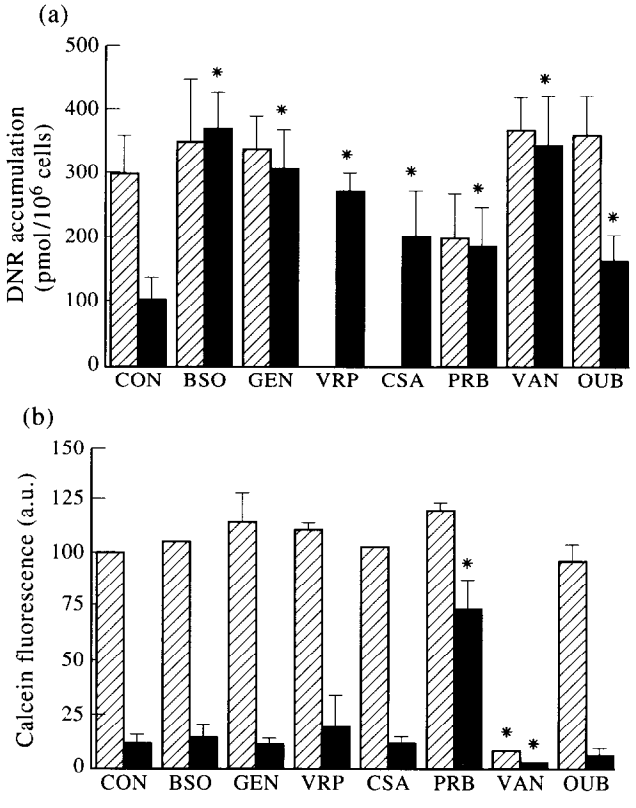

Figure 3. Cellular accumulation of (a) daunorubicin or (b) calcein fluorescence in COR-L23/P (hatched bars) or COR-L23/R (solid bars) cells exposed either to [^{3}H]DNR or calcein AM. Experiments were carried out in normal medium or in medium to which various chemical modulators had been added as described. CON, control; BSO, buthionine sulphoximine (25 μM); GEN, genistein (200 μM); VRP, verapamil (10 μM); CsA, cyclosporin A (4.2 μM); PRB, probenecid (5 mM); VAN, orthovanadate (1 mM); OUB, oubain (1 mM). Data are means (S.D.) from three independent experiments. Calcein fluorescence was normalised to 100% in COR-L23/P cells exposed under control conditions. Asterisks indicate values which were significantly different ($P \leq 0.02$, Student's test) from the respective control. Reproduced from Versantvoort CHM, *et al.* 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* 1995, Vol. 63, pp. 855–862. Reprinted by permission of John Wiley & Sons, Inc.

CONCLUSION

There are certain to be rapid advances in our knowledge of the biology of MRP over the next couple of years. These will

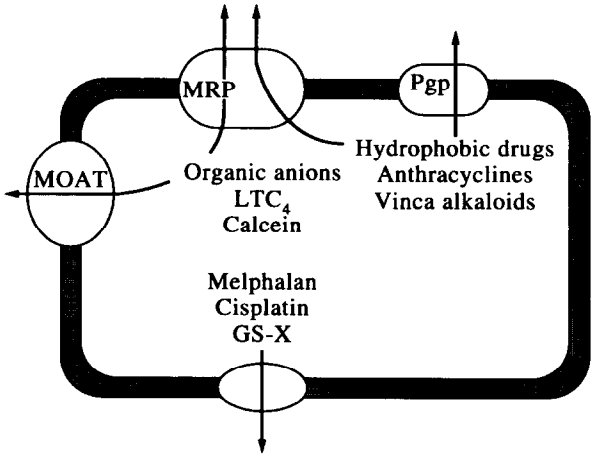

Figure 4. Diagram of a cell showing the operation of various transport mechanisms related to multidrug resistance. MRP is clearly a MOAT (multispecific organic anion transporter) but it is not clear whether or not other MOATs also exist. The relationship between the glutathione conjugate (GS-X) transporter described by Ishikawa and colleagues [61] and MRP is currently uncertain.

include further understanding of its role in normal tissues and the significance of high-level expression in various tumour types. It currently appears unlikely that any strategy designed to suppress efflux of cytotoxic drugs by a mechanism involving MRP will not produce resultant increases in normal tissue toxicity. The relationship between MRP-associated transport of lipophilic cytotoxic drugs and transport of organic anions will hopefully soon be elucidated and it will become clearer as to whether or not MRP actually contains drug-binding sites. Such knowledge may lead to a more systematic approach to the possibility of modulation of MRP action in a manner resulting in potential therapeutic advantage. As in P-glycoprotein-mediated resistance, development of better modulators will probably involve identification in structure/activity series of analogues of "off-the-shelf" compounds found to possess desirable properties.

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