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Original Paper

Dipyridamole-mediated Reversal of Multidrug Resistance in MRP Over-expressing Human Lung Carcinoma Cells *In Vitro*

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Expression of the multidrug resistance-associated protein (MRP) is widespread in human malignancies, high levels are associated with poor prognosis and may be responsible for intrinsic and radiotherapy-induced chemoresistance. In this study, the nucleoside transport inhibitor, dipyridamole (DP), was investigated as a chemosensitiser of MRP. In growth inhibition assays MRP-overexpressing COR L23/R cells were 20 times more resistant to VP16 and doxorubicin compared with the parental COR L23/R human lung carcinoma cells. DP caused an approximately 8-fold sensitisation of the resistant cells and a 2-fold sensitisation of the parental cells. DP enhanced the accumulation of VP16 1.5 to 2-fold in the parental cells, but had only a modest effect on VP16 accumulation in the resistant cells. VP16 efflux was rapid in both cell lines. DP caused a modest and transient inhibition of the initial efflux in the resistant cells but not the parental cells. Incubation with DP caused a progressive decrease in GSH levels which was more rapid and profound in COR L23/R cells than in COR L23/P cells. Thus, chemosensitisation to VP16 by DP in MRP-overexpressing COR L23/R cells appears to be caused by depletion of cellular GSH rather than a direct effect of DP on MRP-mediated drug accumulation and efflux. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: dipyridamole, multidrug resistance-associated protein (MRP), VP16 (etoposide), resistance modulation, glutathione
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INTRODUCTION

MULTIDRUG RESISTANCE (MDR) severely compromises the efficacy of cancer chemotherapy in the clinic. It occurs when tumours, that may initially have been sensitive, become resistant to a variety of therapeutically important anticancer drugs that are structurally unrelated and have diverse subcellular targets. *In vitro* studies have shown that MDR is accompanied by reduced intracellular drug accumulation due to increased drug efflux by energy dependent trans-membrane drug transport proteins [1]. The first of these to be identified was the p170 glycoprotein (Pgp), the product of the *mdr1* gene. Overexpression of Pgp has been demonstrated in many cells with the MDR phenotype [1]. Studies of a Pgp negative multidrug resistant cell line led to the identification of another transmembrane protein, the p190 multidrug resistance-associated protein (MRP) [2]. Subsequent studies have shown that transfection of the *mrp* gene confers drug resistance [3]. Conversely deletion of *mrp* is associated with increased sensitivity to anticancer drugs [4, 5]. Despite their similar function, MRP and Pgp show little sequence homology, which is restricted to the ATP binding site. Drug cross-resistance profiles are similar but not identical for MRP and Pgp overexpressing cells [6] and agents that reverse Pgp are usually less effective on MRP.

The clinical relevance of MRP overexpression is uncertain but recent evidence has indicated that MRP is commonly found in cells derived from solid tumours; in one study MRP was detected in 54/55 solid tumour cell lines [7] and MRP expression has been reported in 46/60 cells in the NCI screen compared with only 14/55 expressing Pgp [8]. The pre-

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valence of MRP in cell lines not subjected to laboratory drug selection suggests MRP may be widespread in human malignancies and possibly responsible for intrinsic drug resistance in chemo-naïve tumours. Studies *in vitro* showing increased MRP expression following radiation suggest that MRP may also be responsible for drug resistance that often follows radiation treatment [9]. High levels of MRP have been found in some cell lines derived from tumours that characteristically respond poorly to chemotherapy, e.g. lung cancer and melanoma, and patients with tumours expressing high levels of MRP have significantly worse survival following chemotherapy [10, 11]. MRP may also be an indicator of poor prognosis, for example, in breast cancer MRP expression was associated with an increased risk of relapse [12] and in neuroblastoma there is a significant correlation between the levels of MRP and *N-MYC* amplification, which is in turn a highly significant indicator of a poor prognosis [13].

Owing to the widespread occurrence in human tumours, there is considerable interest in identifying ways in which MRP-mediated resistance can be reversed. Inhibitors of Pgp can resensitise MDR cells and many studies have been conducted into the use of Pgp inhibitors as modulators of MDR [14]. The Pgp reversing agent, verapamil, can modulate resistance in MRP overexpressing cells. Other agents, such as nicardipine and genistein can also reverse MRP associated resistance, but reversal is only partial even at highly toxic concentrations [6]. The precise function of MRP remains to be determined, but it is considered to be an organic anion transporter, with reduced glutathione (GSH) or glutathione conjugates, including leukotriene C_4, being the natural substrates, and the transport of glutathione- glucuronate- or sulphate- conjugates of cytotoxic drugs is considered to be responsible for MRP-mediated MDR [15]. Thus, both probenicid (an organic anion transport inhibitor) and MK571 (an LTC_4 receptor antagonist) can reverse MRP mediated drug resistance [16, 17].

Dipyridamole (DP), because of its known interaction with nucleoside transport [18], has been most extensively studied as an augmentor of antimetabolite cytotoxicity [19]. Nevertheless, DP has been shown to increase the intracellular concentration and cytotoxicity of a variety of MDR drugs [19]. There is good evidence to show that this is mediated via an interaction of DP with Pgp [20]. DP may also be inhibiting other drug resistance mechanisms, particularly in the case of resistance to VP16 (etoposide). In the drug-sensitive 2008 cell line, DP has been shown to decrease the efflux of VP16 and increase the intracellular accumulation, thereby potentiating the cytotoxicity [21]. In other studies, DP increased the intracellular concentration of VP16 not only in mdr-transfected cells but also parental cells [22]. Similarly, DP enhanced the accumulation and potentiated the cytotoxicity of VP16 to the same extent in parental and Pgp overexpressing CHO cells [23], suggesting that DP augmentation of VP16 in these cells may be mediated through inhibition of another drug efflux mechanism, possibly MRP.

The aim of the current study was to determine if the sensitisation to VP16 by DP is due to inhibition of MRP using parental and MRP overexpressing human lung cancer cells. The effect of DP on VP16 growth inhibition and accumulation was investigated in parental, COR L23/P cells and the MRP overexpressing sub-line, COR L23/R. DP was used at a concentration of 10 μM to allow comparison with previous studies on the effect of DP on VP16 accumulation, retention and growth inhibition in other cell lines [21, 23]

MATERIALS AND METHODS

Drugs and chemicals

VP16 (etoposide), doxorubicin, DP, tissue culture reagents, glutathione reductase and routine chemicals were obtained from Sigma Chemical Company (Poole, Dorset U.K.) unless stated otherwise. Stock solutions of VP16, doxorubicin and DP were made by dissolving them in dry DMSO. [^{3}H]-VP16 (1.1 Ci/mmol) in ethanol was obtained from Moravek Biochemicals (Brea, California U.S.A.) and [U-^{14}C] sucrose, 460-740 mCi/m mol was obtained from Amersham (U.K.). Tissue culture plastic-ware was obtained from Nunc Life Technologies (Paisley, U.K.).

Cell lines

The human large cell lung cancer cell line COR L23/P and its doxorubicin-selected resistant derivative, COR L23/R, described previously [24–26] were a gift from P. Twentyman (MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge U.K.). The resistant cells overexpress MRP but not Pgp. They were adapted to grow in HEPES-supplemented RPMI 1640 medium + 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C. COR L23/R cells were maintained in the presence of 0.2 μg doxorubicin/ml but washed free of drug and cultured in drug free medium for 2–5 days prior to use in experiments described below. Cells were examined for mycoplasma by fluorescence microscopy following fixation and staining with Hoechst 33258 at monthly intervals and found to be negative.

Growth inhibition assays

Cells were seeded at 1 to 1.5×10^3 cells/well in 100 μl medium in replicate 96-well plates (leaving the outer wells with 100 μl medium alone to minimise 'edge effect') and allowed to attach overnight. After 16–24 h the medium was replaced with that containing varying concentrations of VP16 with or without 10 μM DP in a final DMSO concentration of 0.1%; 10 replicate wells were used for each drug concentration. A replicate plate was fixed as described below to obtain an estimate of the cell density at the start of the drug incubation. The plates were incubated for 6 days at 37°C before assaying for cell growth by minor modification of a previously described method [27]. Briefly, the 96-well plates were rinsed in Dulbecco's modified PBS (DulA: Gibco, Paisley, U.K.) and fixed in Carnoys fixative (methanol: acetic acid, 3:1), rinsed in tap-water, air dried and stored at 4°C until stained. The plates (together with the pre-incubation plate) were stained with 0.4% (w/v) sulforhodamine B in 1% acetic acid (100 μl/well) for 30 min then rinsed 3x in 1% acetic acid to remove unbound stain. Protein-bound stain was extracted using 100 μl 10 mM Tris base per well. The optical density of the wells was read on a computer-interfaced MR700 microtitre-plate reader (Dynatech, Billingshurst, U.K.) relative to an air blank using a 570 nm filter. Cell growth was expressed as a percentage of the growth in appropriate controls i.e. 0.1% DMSO or 10 μM DP. The IC_{50} values were determined by non-linear regression curve fitting using GraphPad PRISM™ (San Diego, California, U.S.A.) software.

Measurement of [^{3}H]VP16 accumulation

In order to mimic the conditions for growth inhibition as closely as possible, the accumulation of VP16 was measured in complete medium rather than buffer. Cells were harvested without trypsin, using 0.4% (w/v) EDTA in Dul A, to avoid

the proteolytic digestion of plasma membrane proteins. The cells were resuspended at 2×10^7/ml in complete medium containing 0.1% DMSO with or without 10 μM DP. [^{3}H] VP16 was added to give a final concentration of 10 mg/ml (17 μM) and mixed vigorously. In some experiments 0.5 μCi [^{14}C]-sucrose/ml was also included to correct for trapped extracellular medium. The cells were agitated at room temperature and triplicate 50 μl (i.e. 10^6 cells) samples were spun through 100 μl silicone oil, specific gravity 1.028 (Dow-Corning 556:550 9:11; BDH, Merck Ltd, Lutterworth, Leicestershire, U.K.) overlaying 50 μl 3 M KOH in microfuge tubes (0.4 ml, BDH) at intervals. The tubes were capped and cut in the silicone layer, the lower portion, containing the cells lysed in KOH, was placed in scintillation vials. The radioactivity was measured, following neutralisation of the cell lysate with 0.25 M acetic acid, by scintillation counting on a LKB-Wallac S1410 β-counter. Viable cell counts (trypan blue exclusion) were taken at intervals during the accumulation period.

Measurement of [^{3}H]VP16 retention

Cells, harvested as described for accumulation experiments, were resuspended at 2×10^7/ml in complete medium containing 10 mg/ml (17 μM) [^{3}H]VP16 and agitated for 20 min at room temperature. In some experiments 0.5 μCi [^{14}C]-sucrose/ml was also included to correct for trapped extracellular medium, as above. Triplicate 50 μl (i.e. 10^6 cells) samples were spun though oil (as described above) to determine the initial intracellular VP16 concentration and the remaining cell suspension was pelleted and resuspended in an equal volume of fresh medium with or without 10 μM DP and mixed thoroughly. The cells were agitated at room temperature and triplicate 50 μl samples were spun through oil into 3 M KOH to lyse the cells at intervals. The intracellular [^{3}H] was measured as described for the accumulation experiments. Viable cell counts (trypan blue exclusion) were taken at intervals during the efflux period.

Measurement of intracellular reduced glutathione

Cells were seeded into replicate 60 mm diameter dishes at 3×10^5/dish in drug-free medium. After 3 days the medium was replaced with that containing 0.1% DMSO ± 10 μM DP. Following the desired incubation period, cells were harvested by trypsinisation, pelleted and resuspended at 2×10^6 cells/ml in 0.1% Triton X 100 in Dul A. After 30 min duplicate 165 μl aliquots were frozen at −80°C until assayed for GSH and protein content. Protein estimations were made using Coomassie brilliant blue protein assay kit (Pierce and Warriner, Chester, U.K.) by adapting the standard protocol for use with microtitre plates. Standards of 25 to 750 μg/ml were made by diluting the 2 mg BSA/ml solution provided with 0.1% Triton-X 100. Duplicate 5 μl aliquots of standards and cell lysates were dispensed per well of a microtitre plate, 125 μl of the Coomassie reagent was added and the plates read at 595 nM on a Dynatek MR700 plate reader. The protein concentration of the samples was estimated from the standard curve. The glutathione content of the cell lysates was determined by a microtitre plate enzymatic recycling assay adapted from a previously described method [28] by Kearns and Hall [29] which utilises the formation of a coloured product from 5,5′-dithio-*bis*(2-nitobenzoic acid) with GSH. Both reduced (GSH) and oxidised (GSSG) forms were assayed simultaneously by the inclusion of glutathione reductase and NADPH in the reaction buffer. Standards of 1–20 μM GSSG were made by dilution of 1 mM stock solution. Reaction buffer was made up fresh immediately before the assay as follows: 0.1 M potassium phosphate buffer pH 7.0 + 1 mM EDTA, glutathione reductase 2.4 U/ml potassium phosphate buffer, 1.5 mM 5,5′ -dithio-*bis*(2-nitobenzoic acid) in 0.5% $NaHCO_3$ and NADPH 4 mg/ml in 0.5% $NaHCO_3$ in the ratio 15:1:1:1. Eight 20 μl sample blanks and duplicate 20 μl aliquots of standards and cell lysates were dispensed into individual wells of a 96 well plate, the reaction was initiated by the addition of 180 μl reaction buffer to each well and the plates read immediately and at 2 min intervals for 20 min at 412 nm on a Titertek Multiskan MCC/340 Mk II microtitre plate reader (Labsystems, Basingstoke, U.K.). The curve of the change in absorbance against the concentration of the standards was linear, the GSH equivalents being twice the GSSG concentration. Sample GSH concentrations were estimated from a comparison of the change in sample absorbance with the standard curve.

Statistical analysis

Determination of a significant difference between data sets was assessed using the Student's *t*-test (GrapPad Instat software).

RESULTS

Growth inhibition assays

The effect of 10 μM DP on the growth inhibition by VP16 and doxorubicin are shown in Figure 1. Comparison of the IC_{50} data from several experiments (Table 1) shows that the COR L23/R cells were approximately 20 times more resistant to VP16 and approximately 10-to 20-times more resistant to doxorubicin than the parental cells. DP enhanced the growth inhibitory effects of both drugs in the parental and resistant cell lines but whereas there was only approximately a 2-fold potentiation of the parental cells, there was around an 8-fold potentiation of the resistant cells. DP largely restored the sensitivity of the resistant cells to VP16 since although the IC_{50} for VP16 + DP in the resistant cell line (mean 220 nM) was still approximately with 2-fold higer compared with the IC_{50} for VP16 alone in the parental cells (mean 460 nM) the difference was not significant ($P = 0.09$: paired Student's t-test). All results were normalised by comparison with either a 0.1% DMSO control or 10 μM DP alone control, but on comparing these two controls, it was noted that DP alone had a growth inhibitory effect and this was significantly greater in the resistant than in the parental cells; the growth of cells exposed to DP was 78 ± 13% and 64 ± 8% of the DMSO control in the parental and resistant lines, respectively (6 observations each, $P = 0.049$).

VP16 accumulation and retention

The effect of DP on the accumulation and efflux of VP16 was measured in the cell lines to determine if this was responsible for the potentiation of VP16 growth inhibition by DP. Substantial intracellular radiolabel was detected at the earliest time point, approximately 0.5 min (Figure 2), this was also observed in experiments where data were corrected for trapped intercellular fluid, by the inclusion of [^{14}C]-sucrose, (Figure 2, inset). In parental cells, VP16 accumulation approximately doubled in the first 20 minutes and by this time steady-state levels had been achieved and no further accumulation took place. Co-incubation with DP enhanced

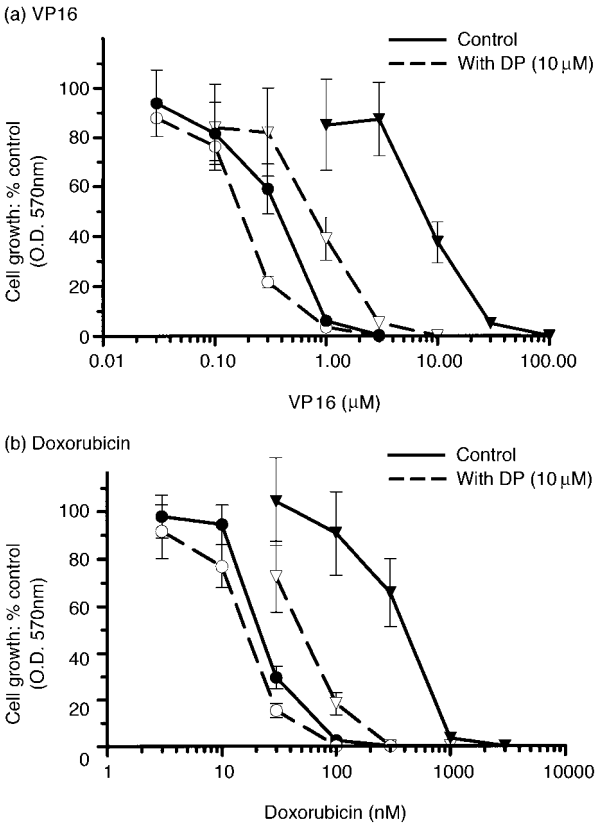

Figure 1. The effect of dipyridamole (DP) on the inhibition of COR L23/P and COR L23/R cell growth by VP16 and doxorubicin. Cell growth was determined after 6 days continuous exposure to increasing concentrations of VP16 (a), or doxorubicin (b) with or without co-exposure to 10 μM DP by sulphurhodamine B assay. Data are COR L23/P, circles; COR L23/R, triangles, mean ± S.D. (standard deviation) of pooled data from 3 independent experiments.

Table 1. *Comparison of the growth inhibition IC_{50} data from parental and resistant cells exposed to VP*16 *and doxorubicin ± DP*

	IC_{50} nM		
	COR-L23/P	COR-L23/R	Resistance factor
VP16	460 ± 90	8720 ± 770	19.5 ± 4.6
VP16 + DP	220 ± 20	1120 ± 500	5.2 ± 2.2
Enhancement factor	2.1 ± 0.3	8.5 ± 2.5	
Doxorubicin	24, 62	476, 573	20, 9.2
Doxorubicin + DP	19, 23	59, 81	3.1, 3.5
Enhancement factor	13, 2.7	8.1, 7.1	

Resistance factor calculated from: $\frac{IC_{50}\text{ VP16 or doxorubicin in COR L23/R cells}}{IC_{50}\text{ VP16 or doxorubicin in COR L23/P cells}}$ for each independent experiment where parental and resistant cells were assayed together. Enhancement factor calculated from: $\frac{IC_{50}\text{ VP16 or doxorubicin alone}}{IC_{50}\text{ VP16 or doxorubicin+DP}}$ for each independent experiment where growth inhibition by drug alone and drug + DP were assayed together. Figures are mean ± standard deviation (S.D.) for 3 experiments (VP16) or individual values from two experiments (doxorubicin).

VP16 accumulation and steady state levels, which were reached after a longer incubation (40 min), were 1.5–2-fold higher than those measured in COR L23/P cells incubated with VP16 alone. In contrast, in the resistant cells, there was no significant increase in intracellular VP16 over the 60 minute incubation in the absence of DP and the steady state levels were 50–65% of those in the parental cells. DP caused only a modest elevation in VP16 levels in the resistant cells.

The detection of higher steady-state intracellular VP16 levels in the presence of DP suggested that either DP stimulates the uptake of VP16 or that there is an efflux mechanism operating to limit intracellular accumulation of VP16 which can be inhibited by DP. Therefore, the effect of DP on [^{3}H]VP16 efflux in pre-loaded COR L23/P and COR L23/R cells was investigated. The intracellular VP16 content was

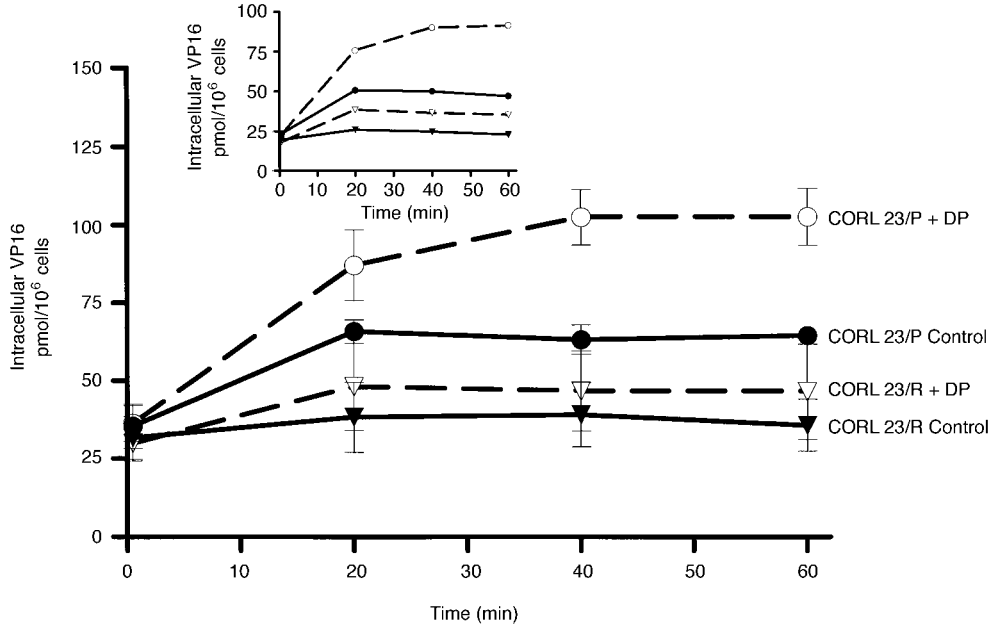

Figure 2. The effect of dipyridamole (DP) on VP16 accumulation in COR L23/P and COR L23/R cells: Cells were harvested using EDTA alone and resuspended in medium containing 17 μM VP16 with or without 10 μM DP before intracellular accumulation was assessed. Mean ± S.D. (standard deviation) of pooled data from 3 independent experiments uncorrected for trapped extracellular VP16 are shown. The insert shows data for a single experiment corrected for VP16 entrapment by inclusion of [^{14}C] sucrose.

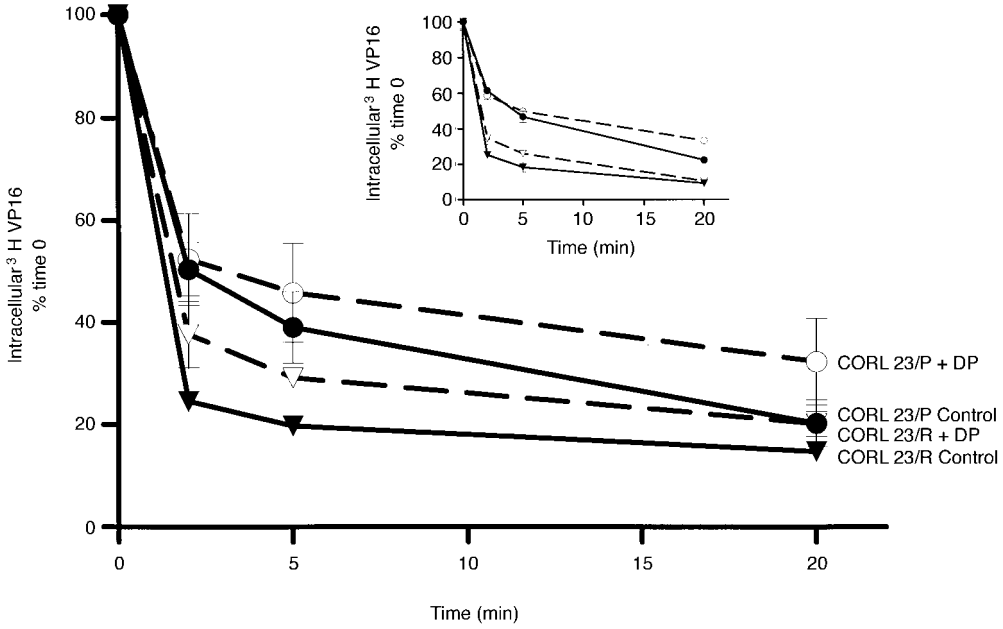

Figure 3. The effect of dipyridamole (DP) on VP16 retention in COR L23/P and COR L23/R cells. Cells were harvested using EDTA alone and resuspended in medium containing 17 μM VP16 for 20 min, pelleted and resuspended in fresh medium with or without 10 μM DP before intracellular retention was determined. Mean ± S.D. (standard deviation) of pooled data from three independent experiments uncorrected for trapped extracellular VP16. The inset shows data for a single experiment corrected for VP16 entrapment by inclusion of [^{14}C] sucrose.

74 ± 14 pmol/10^6 in parental cells and 42 ± 9 pmol/10^6 in resistant cells prior to efflux. In the absence of DP, the initial efflux was very rapid in both cell lines with approximately, 50% and 75% of the intracellular VP16 lost in the first 2 min from parental and resistant cell lines, respectively. Thereafter the rate of efflux was slower and there was little difference in the retention in the two cell lines by 20 min (Figure 3). DP significantly retarded the rapid phase of VP16 efflux in the resistant cells ($P = 0.03$) but not the parental cells, but after 5 min the effect was modest (and not significant) in both cell lines. Data for a single experiment, corrected for entrapped extracellular fluid (by inclusion of [^{14}C]-sucrose) was essentially the same (Figure 3, inset).

Cellular glutathione content

Since glutathione has been implicated in the mechanism of MRP-mediated drug resistance, the effect of DP on the glutathione content was determined in the two cell lines. Although the level of GSH per cell was higher in the parental cells than in the resistant cells (22.8 ± 8.2 for COR L23/P and 16.6 ± 2.8 nM/10^6 cells for COR L23/R), when expressed in relation to cellular protein there was no difference in glutathione concentration between the cells (56.6 ± 10.8 nM/mg protein for COR L23/P and 55.4 ± 9.4 nM/mg protein for COR L23/R). DP caused a significant depletion of cellular GSH in the resistant cells after only 1 h (Figure 4). In contrast, a significant depletion of the levels in the parental cells was not detectable until 24 h.

DISCUSSION

COR L23/R cells were found to be approximately 20-fold more resistant to both VP16 and doxorubicin compared with COR L23/P cells, in agreement with previously reported levels of doxorubicin resistance in these cells [25]. Investigation of the mechanism of VP16 resistance showed that although 20-fold resistant, VP16 accumulation in resistant cells was only 2-fold lower than that in the parental cells. Similar observations have been reported for MRP over-expressing CCRF CEM cells that, despite being 200-fold more resistant to VP16 than parental cells, still accumulate 50% of the parental levels of the drug [30]. Co-incubation with DP caused an approximately 8-fold sensitisation of the resistant sub-line, largely but not entirely overcoming the resistance to VP16 and doxorubicin in COR L23/R cells. DP also caused a 2-fold sensitisation of the parental COR L23/P cells. Thus, in these cells, DP shows superior activity to either PSC 833 or verapamil which only reduced the 58-fold resistance to vincristine in COR L23/R cells 4- and 9-fold,

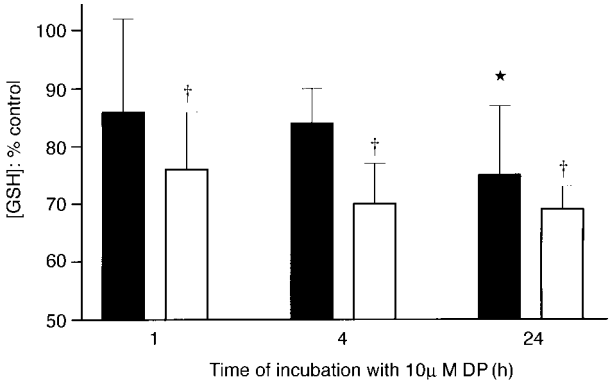

Figure 4. Effect of dipyridamole (DP) on the glutathione content of COR L23/P and COR L23/R cells. The cellular glutathione content of COR L23/P (solid bars) and COR L23/R (open bars) exposed for 1, 4 and 24 h to 10 μM DP in 0.1% DMSO was determined as described in Materials and Methods and expressed as a percentage of the glutathione content of control cultures exposed to 0.1% DMSO. Data were pooled from three independent experiments and significant difference from the control is given by *$P < 0.05$. †$P < 0.01$.

respectively, and this was accompanied by a 2- and 3-fold potentiation of the parental cells [26].

Chemosensitisation by DP was studied by determining the effect of DP on drug accumulation. DP enhanced the accumulation of VP16 1.5 to 2-fold in the parental cells, suggesting that the 2-fold enhancement of the growth inhibitory effects of VP16 in parental cells is mediated by increased intracellular accumulation. DP caused a modest increase in VP16 accumulation in the resistant cells, which was insufficient to overcome the accumulation deficit. Measurement of VP16 efflux showed that, despite a more rapid initial efflux rate in the resistant cells, by 20 min the retention was the same in both cell lines. DP inhibited the initial efflux rate in the resistant cells but not the parental cells, although this inhibition was modest and transient. These data suggest that VP16-resistance in MRP-overexpressing COR L23/R cells is not entirely due to reduced intracellular accumulation and retention of drug and that chemosensitisation by DP cannot be attributed to increased intracellular levels in the drug-resistant cells. A similar sensitisation to VP16 by verapamil has been observed in MRP overexpressing CCRF CEM cells in the absence of enhanced drug accumulation [30]. One possible explanation could be sequestration of the cytotoxic drug at an intracellular site distinct from its nuclear target in the drug resistant cells. Studies using the fluorescent drug, daunorubicin, have demonstrated the presence of nuclear staining in the parental COR L23/P cells but the absence of nuclear fluorescence in the 16-fold resistant COR L23/R cells [26]. In the presence of verapamil, which caused a 4-fold sensitisation of the resistant cells, daunorubicin fluorescence was detected in the nucleus of COR L23/R cells. There may be similar differences in intracellular VP16 disribution in COR L23/R cells that may be normalised in the presence of DP.

The precise function of MRP in mediating MDR remains to be elucidated. In MRP overexpressing cells sensitisation is not necessarily accompanied by changes in accumulation, suggesting MRP is not simply a drug efflux pump like Pgp. The reversal of resistance is accompanied by GSH depletion, not only by buthionine sulphoximine (BSO) [31] and other inhibitors of GST metabolism [32] but also by other MRP reversing agents [17, 30] Chemosensitisation to daunorubicin and vincristine in COR L23/R cells has been shown to be accompanied by decreased drug efflux and increased accumulation [17]. In contrast, enhancement of VP16 cytotoxicity in MRP-overexpressing CCRF CEM cells through BSO-mediated GSH depletion was demonstrated to occur in the absence of increased drug accumulation [30]. Since DP reversed VP16 resistance in COR L23/R cells without overcoming the accumulation deficit, its effect on cellular GSH was investigated. Incubation with DP caused a progressive decrease in GSH levels which was more rapid and profound in the resistant cells. In the study reported here, the 30% decrease in cellular GSH in CORL 23/R cells was accompanied by an 8-fold sensitisation to VP16. In previous studies demonstrating verapamil-mediated sensitisation of COR L23/R cells to daunorubicin (4-fold) and vincristine (9-fold) [26] and CCRF CEM cells to VP16 (2.5-fold) [30] GSH was depleted by 60–68% [17, 30]. Similarly BSO, which reduced cellular GSH levels in COR L23/R cells by 70% caused an 11-fold sensitisation to vincristine [31]. Thus, sensitisation of MRP-overexpressing cells cannot entirely be explained on the basis of the extent of GSH depletion. Nevertheless, chemosensitisation to VP16 by DP in MRP-overexpressing COR L23/R cells appears to be associated with depletion of cellular GSH rather than a direct effect of DP on MRP-mediated drug accumulation and efflux.

Depletion of GSH by BSO has been found to be more cytotoxic to MRP over-expressing HL60/ADR and NIH3T3 MRP-transfectants than the wild-type cells [33] but less cytotoxic to drug resistant COR L23/R cells [31]. In the studies reported here depletion of GSH by DP was accompanied by significantly greater growth inhibition by DP in the resistant COR L23/R cells than in the parental cells.

DP is primarily considered to be an inhibitor of nucleoside transport, but it has been shown to inhibit other transport mechanisms including Pgp [20] and cisplatin transport [34] without affecting membrane fluidity or permeability [34, 35]. DP has also been shown to inhibit methotrexate efflux [36] and recently methotrexate has been identified as a substrate for MRP [37]. The reversal of VP16 and doxorubicin resistance by DP in COR L23/R cells suggests DP modulates MRP-mediated resistance and this may be responsible for the previously observed inhibition of methotrexate efflux. Although chemosensitisation of COR L23/R cells by DP appears to involve GSH depletion the precise mechanism is as yet unclear. This report is the first demonstration of the depletion of GSH by DP and the mechanism for this remains to be elucidated. DP interacts with a number of plasma membrane transport proteins and it may, therefore, inhibit γ-glutamyl transpeptidase, the plasma membrane associated GSH salvage enzyme. Alternatively, DP uptake into cells has been demonstrated [38], whereupon it may inhibit glutathione synthetic enzymes. The understanding of the role of GSH in MRP-mediated drug resistance is important as the lack of a correlation between accumulation and cytotoxicity makes it difficult to identify chemosensitisers on the basis of assays measuring drug accumulation/efflux.

MRP expression is a frequent occurrence in solid tumours and may be associated with intrinsic resistance to chemotherapy and poor prognosis. Although MRP mRNA is also frequently found in normal tissues [7], the recent generation of viable MRP-knockout mice [5] suggests that inhibition of MRP may not necessarily affect whole animal (or patient) toxicity and it, therefore, remains a viable target for the modulation of chemoresistance. Although the work reported here awaits confirmation in other MRP-overexpressing cells the finding that DP reverses drug resistance in COR L23/R cells, and the earlier observation that DP reverses MDR in Pgp overexpressing cells [19], indicates that DP may be a valuable tool in circumventing drug resistance in tumours expressing MRP and/or Pgp.

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