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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

M.A. Barrand, T. Rhodes, M.S. Center and P.R. Twentyman

The doxorubicin-selected multidrug resistant (MDR) human large cell lung cancer line COR-L23/R, lacks P-glycoprotein but shows a drug accumulation deficit. It does however overexpress a 190k membrane protein which shares an epitope with, but is otherwise distinct from, P-glycoprotein. The resistant cells show only a small sensitisation to vincristine and daunorubicin on treatment with cyclosporin A and its more potent analogue, PSC-833 despite an increase in drug accumulation. Verapamil, another effective resistance modifier in P-glycoprotein MDR cells, is slightly more effective. Fluorescent daunorubicin distributes in the cytoplasm and nucleus of sensitive parent COR-L23 cells but is confined to cytoplasmic perinuclear vesicles in resistant cells. Addition of cyclosporin A or PSC-833 slightly increases cytoplasmic fluorescence whereas verapamil also increases nuclear fluorescence. Resistance in this non-P-glycoprotein MDR line, COR-L23/R where these resistance modifiers have little effect may be associated with expression of the 190k protein.
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INTRODUCTION

DECREASED DRUG accumulation is one of a variety of mechanisms which have been shown to account for the multidrug resistance observed in tumour cells in culture. Hyperexpression of the drug efflux pump, P-glycoprotein, has been associated in many cases with this decrease in drug accumulation [1]. However there are now an increasing number of reports of multidrug resistant (MDR) cells that show decreased accumulation of drugs of the type normally expelled by the efflux pump but that do not express *mdr* mRNA or contain P-glycoprotein [2–5]. One such resistant cell line has been developed from a human large cell lung cancer cell line, COR-L23. The MDR variant, COR-L23/R was derived by continuous *in vitro* exposure to doxorubicin [6] and shows cross resistance to vincristine and to colchicine [7]. It does not hyperexpress the *mdr* 1 gene as determined by northern blot analysis [8] and shows no evidence of the *mdr* gene product, P-glycoprotein, either from western blot analysis using a single anti-P-glycoprotein monoclonal antibody, C219 [8] or from immunocytochemical staining using a panel of anti-P-glycoprotein antibodies, JSB-1, C219 and MRK16 [9]. The mechanisms underlying the chemoresistance and decreased drug accumulation observed in these non-P-glycoprotein MDR cells are unknown, but we provide evidence here that there is overexpression in our resistant cells of a membrane protein slightly larger in size than P-glycoprotein (approximately 190k), but containing a short amino acid sequence antigenically similar to a region of P-glycoprotein close to one of its nucleotide binding sites.

It has been shown that at least partial reversal of resistance in MDR cells containing P-glycoprotein may be brought about by verapamil and by cyclosporins [10], this reversal being associated usually but not invariably with reduction in the drug accumulation deficit. There is evidence to suggest that these resistance modifiers may exert their actions at least in part by binding directly to P-glycoprotein so reducing its drug efflux capability [10]. The actual sites for binding of each modifier to the protein

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may be different and in addition the effects of cyclosporins on resistance may be more complex than those of verapamil [10, 11]. Nevertheless these agents have the potential to assist in overcoming the resistance to chemotherapy which has been observed clinically. However, it has been revealed from *in vitro* studies that such resistance modifiers may be ineffective in enhancing chemosensitivity in those MDR cells that do not express P-glycoprotein [12–14]. We have compared the effects of the resistance modifiers, cyclosporin A, the more potent but non-immunosuppressive analogue PSC-833 [15] and verapamil not only on chemosensitivity but also on drug accumulation and intracellular distribution of vincristine and daunorubicin in our MDR cell line that does not contain P-glycoprotein but which hyperexpresses another 190k membrane protein. Preliminary results of this work have already been presented [16].

MATERIALS AND METHODS

Cell lines

The cells used in this study were of the parental large cell lung carcinoma cell line, COR-L23/P (L23/P) and its MDR variant COR-L23/R (L23/R) which was derived by continuous exposure of the parent line to increasing concentrations of doxorubicin as described previously [6]. Cells of the small cell lung carcinoma line, NCI-H69 (H69/P) and its P-glycoprotein-expressing doxorubicin-selected MDR variant, H69/LX4 [6], were used as negative and positive controls in the western blot analysis studies. The cells were cultured in RPMI 1640 medium (Gibco Biocult) supplemented with 10% fetal calf serum (Seralab Ltd) together with penicillin and streptomycin (at concentrations of 100 IU/ml and 100 μg/ml, respectively). The L23/P and L23/R cells grow as attached monolayers on plastic whilst the H69/P and H69/LX4 cells grow as floating aggregates. The MDR variant L23/R was maintained in the presence of 0.2 μg/ml doxorubicin but the cells were washed free of drug and kept in drug-free medium for at least 48 h before use in experiments.

Protein separation and immunoblotting

Cells in exponential growth were washed and scraped from their growing surface into phosphate-buffered saline (PBS) containing phenylmethylsulphonyl fluoride (PMSF) at 100 μg/ml, pelleted at 60 *g* for 5 min and then lysed in 1 mmol/l Tris at pH 7.4 containing PMSF. Nuclei and unbroken cells were removed from the homogenate by centrifugation at 450 *g* for 10 min at 4°C and the cell membranes then separated from the resultant supernatant by centrifugation at 60 000 *g* for 1 h at 4°C. Membrane proteins (10–50 μg per well) were dissolved in 0.4% SDS in 0.015 mol/l Tris at pH 6.5 containing 2% β-mercaptoethanol and resolved by electrophoresis in 7.5% polyacrylamide and electroblotted onto nitrocellulose. The filters were exposed for 24 h at 4°C firstly to blocking buffer containing 5 mmol/l EDTA, 0.25% gelatin, 0.01 mol/l NaN_3, 0.15 mol/l NaCl, 0.05 mol/l Tris at pH 7.4 and 0.05% Nonidet P40, secondly to rabbit antiserum ASP14 raised against the synthetic peptide sequence GTQLSGGQKQRIAIA [17] diluted 1:400 in blocking buffer and finally with ^{125}I-labelled protein A diluted 1:1000 in blocking buffer before autoradiography.

Chemosensitivity testing

The assay used was based on that originally described by Mosmann, and modified in this laboratory. Cells were plated into 96-well microtitre plates (Falcon Plastics, Cambridge, U.K.) at 1×10^3 (L23/P) and 2×10^3 (L23/R) per well, in 200 μl medium. After a period of approximately 2 h incubation (8% CO_2, 92% air, 37°C) 20 μl of the appropriate concentrations of drug or of vehicle alone were added. In sensitisation experiments, cells were incubated for 1 h with sensitiser before addition of drug. Plates were then incubated for a period of 6 days ('continuous exposure') in a gassing incubator (8% CO_2, 92% air, 37°C). At the end of the incubation period, 20 μl of a 5 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT, Sigma, Poole, U.K.) in PBS was added to each well and the plates returned to the incubator for a further 5 h. The bulk of the medium was then removed from each well, 200 μl of dimethyl sulphoxide (BDH, Poole, U.K.) was added per well and the plates were agitated on a plate shaker for 10 min. Optical densities were read at 540 nm and at a reference wavelength of 690 nm on a Titertek Multiskan MCC ELISA plate reader (Flow Laboratories, Rickmansworth, U.K.). Results were expressed as a fraction of control absorbances.

Drug accumulation studies

[^{3}H]Vincristine hydrochloride (specific activity 244 GBq/mmol) and [^{3}H]daunorubicin (specific activity 51.8 GBq/mmol) were obtained from Amersham International. Accumulation of drug was estimated as follows: briefly, 96 h before experiments, cells were inoculated into wells on six-well multiplates in numbers sufficient to provide equal numbers of cells per well at the time of the experiments, i.e. initially 2×10^4 per well for L23/P and 3×10^4 per well for L23/R. The resistant cells were grown in the absence of selecting drug during this 96 h period. At the start of the experiments the growth medium was replaced in each well with new medium containing labelled drug at concentrations of 0.02 μg/ml (0.04 μmol/l) for daunorubicin and 0.014 μg/ml (0.015 μmol/l) for vincristine and the cells incubated at 37°C for varying times. To terminate uptake, the incubation medium was removed, the cells were washed rapidly three times with ice cold PBS and then lysed over the course of 2 h in distilled water, 0.5 ml volumes being used per well. The contents of each well was transferred to a scintillation vial, mixed with 5 ml Quicksafe A (Zinsser Analytic, Maidenhead, Berks, U.K.) and counted on a Beckman LS 5000 CE liquid scintillation counter following 24 h at room temperature to allow chemiluminescence to have decayed. Cell counts were carried out on sample wells not used for drug uptake so that drug values could be estimated as accumulation per cell.

Visualisation of intracellular drug distribution by confocal microscopy

Single cell suspensions resulting from trypsin/versene treatment and containing a 5×10^4 cells/ml in 2 ml aliquots were allowed to attach overnight at 37°C onto glass coverslips in six-well multiplates. The growth medium was then removed and cells incubated at 37°C for 2 h with 2 ml aliquots of medium containing daunorubicin at concentrations of 10, 1 and 0.1 μg/ml. When resistance modifiers were included, these were added 30 min prior to addition of daunorubicin. Following a quick rinse in PBS, the coverslips with cells attached were inverted and mounted in PBS on glass slides, the edges being sealed to prevent drying out. The slides were then kept on ice until viewed under the confocal microscope, generally within 10 min of preparation. Fluorescence was observed with the Biorad MRC-600 laserscan confocal microscope using excitation filters allowing light of 515–560 nm wave length and emission filters accepting light of 590 nm and above. Pictures obtained by averaging the images from five separate scans were stored

on disc and photographed from a high intensity screen. For visualisation of the Golgi apparatus, cells were fixed in 2% paraformaldehyde for 15 min at room temperature and exposed for 30 min to fluorescent-labelled wheat germ agglutinin (Sigma) at 100 μg/ml in 0.5% saponin in PBS before being examined under the confocal microscope.

Materials

The cytotoxic drugs used were daunorubicin (May & Baker, Rhône-Poulenc U.K., Dagenham, U.K.) and vincristine (Lederle Laboratories, Gosport, U.K.). The resistance modifiers, cyclosporin A and PSC-833 were kindly supplied by Sandoz, Basel, Switzerland and verapamil was obtained from Abbot Laboratories Ltd, Queenborough, Kent, U.K.

RESULTS

Identification of P190 expression

Crude membranes prepared from L23/R and from L23/P cells were subjected to SDS gel electrophoresis and western blot analysis with antiserum ASP14. Following autoradiography, a band corresponding to a molecular weight of about 190k was evident in the membranes prepared from the resistant L23/R cells but not in those from the sensitive parent L23/P cells. Membranes prepared from the P-glycoprotein-containing human resistant small cell lung carcinoma line, H69/LX4 and from its sensitive parent line, H69/P were used as positive and negative controls on the same filters. A single band corresponding to a molecular size of about 170 k was evident in the resistant H69/LX4 cell membranes only (Fig. 1). Duplicate filters probed with the anti-P-glycoprotein monoclonal antibody, C219, showed only a single band at 170 k in the H69/LX4 membranes and no band at all in the L23/R membranes in accordance with previous findings [9].

Chemosensitivity testing

Cyclosporin A at 1, 2 and 5 μg/ml produced a slight, apparently dose-dependent increase in sensitivity of the resistant L23/R cells both to daunorubicin and to vincristine (Fig. 2). The highest cyclosporin concentration chosen for study was around the IC_{50} value for cyclosporin A alone estimated over a 6 day period by the MTT assay [18]. IC_{50} values for daunorubicin and for vincristine together with sensitisation ratios and resistance factors obtained for the cell line following treatment with the resistance modifiers at the highest concentration used are shown in Table 1. The non-immunosuppressive cyclosporin analogue, PSC-833, at equimolar concentrations produced increases in sensitivity of similar magnitude to those of cyclosporin A (Fig. 2 and Table 1). In line with results obtained in a previous study [7], verapamil at 3.3 μg/ml brought about a degree of sensitisation in the resistant cell line, L23/R greater than that seen in the parent L23/P cell line so that resistance factors were reduced. Small increases in drug sensitivity were produced in the sensitive parent line, L23/P also by the cyclosporins. In the case of daunorubicin these were to a lesser degree than those seen in the resistant line so that the resistance factors therefore were slightly though not greatly reduced by cyclosporin A or PSC-833.

Drug accumulation

Accumulation of [^{3}H]daunorubicin and of [^{3}H]vincristine in L23/R and L23/P cells was monitored over the course of 120 min. Steady state conditions were obtained with daunorubicin in resistant cells after about 90–100 min. In the majority of

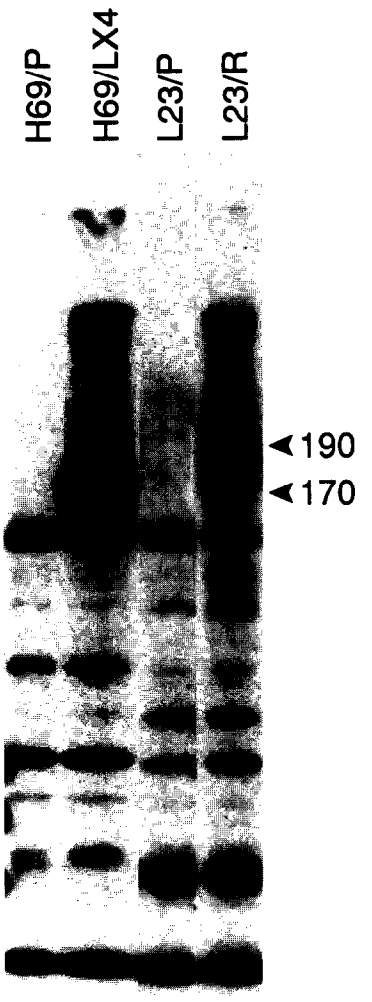

Fig. 1. Reactivity of rabbit antiserum, ASP14, with 50 μg samples of membrane proteins from sensitive H69/P and L23/P cells and resistant H69/LX4 and L23/R cells following SDS gel electrophoresis and western blot analysis. *Ordinate*, molecular weight in thousands.

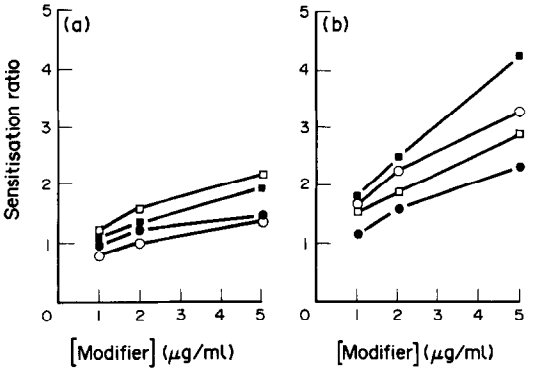

Fig. 2. Effects of different concentrations of cyclosporin A (open symbols) and PSC-833 (closed symbols) on sensitisation of L23/P (circles) and L23/R cells (squares) to (a) daunorubicin and (b) vincristine. Sensitisation ratios were calculated as IC_{50} without modifier/IC_{50} with modifier. Values shown are the mean of ratios calculated from three separate experiments.

Table 1. Effects of cyclosporin A, PSC-833 and verapamil on sensitivity of L23/P and L23/R cells to (a) daunorubicin and (b) vincristine

	Cell line	Modifier: None	Cyclosporin A (5μg/ml)	PSC-833 (5 μg/ml)	Verapamil (3.3 μg/ml)
(a) Daunorubicin					
IC_{50} (μg/ml)	L23/P (sensitive)	0.013 (0.008) $n = 8$	0.010 (0.007) $n = 6$	0.014 (0.005) $n = 3$	0.007 (0.004) $n = 3$
Sensitisation ratio			1.5 (0.1)	1.5 (0.8)	1.5 (0.2)
IC_{50} (μg/ml)	L23/R (resistant)	0.185 (0.118) $n = 8$	0.098 (0.026) $n = 6$	0.145 (0.018) $n = 3$	0.042 (0.016) $n = 3$
Sensitisation ratio			1.9 (0.5)	1.9 (0.5)	4.0 (2.2)
Resistance factor		16 (7)	11 (4)	12 (5)	7.1 (1.6)
(b) Vincristine					
IC_{50} (μg/ml)	L23/P (sensitive)	0.0015 (0.0022) $n = 6$	0.0012 (0.0010) $n = 4$	0.0009 (0.0002) $n = 3$	0.0025 (0.0024) $n = 3$
Sensitisation ratio			3.2 (0.6)	2.3 (0.7)	3.2 (1.3)
IC_{50} (μg/ml)	L23/R (resistant)	0.091 (0.041) $n = 6$	0.033 (0.011) $n = 4$	0.031 (0.005) $n = 3$	0.010 (0.009) $n = 3$
Sensitisation ratio			3.2 (1.2)	4.3 (2.6)	8.9 (4.7)
Resistance factor		58 (31)	49 (27)	37 (18)	21 (15)

IC_{50} values are shown as the mean (S.D. in parentheses) calculated from data obtained in 'n' experiments, each based on determinations from four wells. Sensitisation ratios were calculated as IC_{50} without modifier/IC_{50} with modifier. Resistance factors were calculated as IC_{50} for resistant cells/IC_{50} for sensitive cells.

experiments, the external concentrations of daunorubicin and of vincristine used were close to the IC_{50} values of the sensitive parent population for each drug. Differences in uptake between parent and resistant cells were not apparent immediately. In agreement with previous observations [7], it was found in each of these experiments that there was a lag of 10–60 min before the decreased accumulation seen in the resistant line became evident. The length of this lag phase was dependent on the external concentration of drug ranging from 10 min with daunorubicin at 0.02 μg/ml to 60 min with daunorubicin at 20 μg/ml (data not shown). We have observed such lags in drug accumulation between sensitive and resistant cells in P-glycoprotein-containing resistant cell lines but only when the external drug concentration used exceeded at least 100-fold the IC_{50} for the sensitive cells (unpublished observations). The phenomenon may well be related to saturation of drug transport mechanisms.

Both cyclosporin A at 5 μg/ml and PSC-833 at 5 μg/ml caused a slight but significant increase in the 2 h accumulation of daunorubicin (Fig. 3a) and of vincristine (Fig. 3b) in the resistant cell line, though not to the level of accumulation seen in the sensitive parent cells. Verapamil at 3.3 μg/ml produced a more marked effect on vincristine accumulation than on daunorubicin

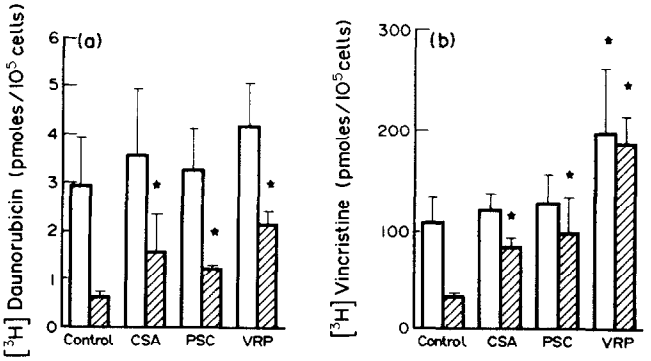

Fig. 3. Accumulation of (a) [^{3}H]daunorubicin and of (b) [^{3}H]vincristine into L23/P (solid bars) and L23/R cells (hatched bars) following exposure to drug alone (control), or to drug in the presence of cyclosporin A (CSA), PSC-833 (PSC) or verapamil (VRP). The values shown are the mean of data derived 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).

accumulation and also affected the parent cells. This modifier actually increased 2 h accumulation of vincristine in the L23/R cells to a level higher than that attained in the parent L23/P cells in the absence of modifier.

Intracellular distribution of daunorubicin

The subcellular distribution of daunorubicin inside the resistant L23/R and sensitive L23/P parent cells following 2 h exposures to external drug concentrations of 0.1, 1 and 10 μg/ml was investigated under the confocal microscope exploiting the natural fluorescence of the anthracycline to visualise the drug. Three different concentrations of drug were used to allow for the possibility of saturation of transport mechanisms involved in drug distribution.

In the L23/P cells following exposure to daunorubicin at 10 μg/ml, i.e. a drug concentration at least 1000 × the IC_{50} for the drug as judged by the MTT assay, intense fluorescence was seen within the nucleus, at the nuclear–cytoplasmic boundary and extensively throughout the cytoplasm both in vesicles and also associated with a network of intermeshed filaments. At the lower daunorubicin concentration of 1 μg/ml, fluorescence was still intense in the nucleus and at the nuclear-cytoplasmic border but in the cytoplasm was confined to vesicles evenly distributed around the nucleus (Fig. 4). At the lowest concentration of daunorubicin used, i.e. 0.1 μg/ml, the distribution of fluorescence was similar to that seen at 1 μg/ml though the fluorescence intensity was weaker both in the nucleus and in vesicles in the cytoplasm. This distribution was unaltered in the presence of either verapamil at 3.3 μg/ml, or cyclosporin A or PSC-833 at 5 μg/ml.

In the resistant L23/R cells following exposure to daunorubicin both at the highest concentration used, i.e. 10 μg/ml and at 1 μg/ml, there was little or no fluorescence detectable inside the nucleus or at the nuclear–cytoplasmic border. As has been noted previously in preliminary work [19], cytoplasmic fluorescence was concentrated into groups of vesicles lying in a distinct area adjacent to the nucleus (Fig. 5a). With daunorubicin at 0.1 μg/ml, it was difficult to detect any fluorescence at all in the resistant cells without enhancing the fluorescence signal by accumulating the images. The fluorescence then visible was essentially all associated with cytoplasmic vesicles distributed as described above. In the presence of cyclosporin A or PSC-833 at 5 μg/ml, there was an increase in fluorescence in the cytoplasm both in vesicles and also spread more diffusely around the nucleus following exposure to 1 μg/ml daunorubicin (Fig. 5b and c). In the presence of verapamil at 3.3 μg/ml, the increase in fluorescence diffusely spread throughout the cytoplasm was somewhat more marked and there was also more fluorescence within the nucleus. However it was still possible to make out the intense fluorescence in the perinuclear region in each cell (Fig. 5d).

In order to identify the nature of these organelles further, L23/R cells previously fixed for 15 min in 2% paraformaldehyde were exposed for 30 min to fluorescent-labelled wheat germ agglutinin at 100 μg/ml in 0.5% saponin in PBS. The fluorescent lectin appeared to concentrate into vesicles lying in a distinct area situated close to the nucleus, a distribution similar to that seen for the fluorescent daunorubicin (Fig. 6).

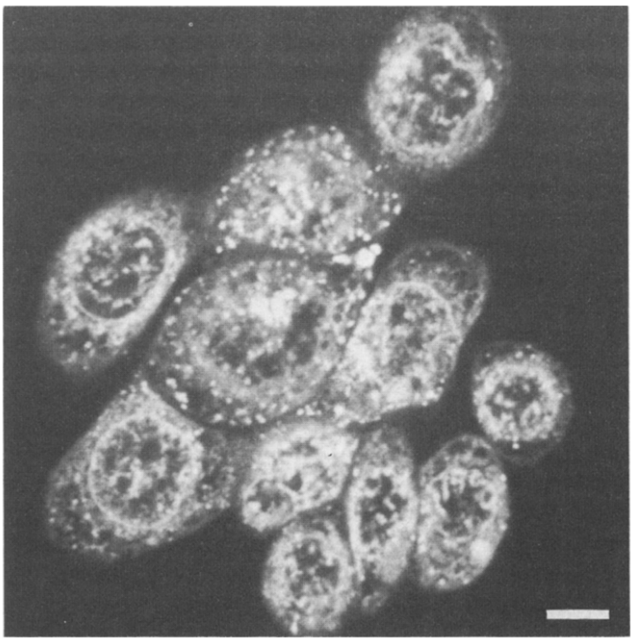

Fig. 4. Subcellular distribution of fluorescence in L23/P cells following 2 h exposure to medium containing daunorubicin at 1 μg/ml. The photograph represents the average of images obtained from five scans. *Bar*, 10 μm.

DISCUSSION

Previous studies have revealed that cells of the L23/R cell line do not express P-glycoprotein, but show a characteristic MDR resistance profile together with a drug accumulation deficit. The protein or proteins responsible for conferring this resistance have not been identified. However we have now detected, in membranes of the resistant variants only, expression of a protein around 190k following western blot analysis using the rabbit antiserum ASP14. This antiserum was one of a series of 13 antisera raised against synthetic peptides whose sequences appear in the P-glycoprotein molecule [17] but was the only one capable of detecting this 190k protein in addition to recognising P-glycoprotein itself. It has now been used to detect 190k proteins in several non-P-glycoprotein-containing MDR lines [20]. The peptide sequence GTQLSGGQKQRIAIA occurs in a region of the P-glycoprotein molecule close to the nucleotide binding site near the C-terminal and is a sequence that is very highly conserved amongst ATPase proteins throughout the Animal Kingdom [21]. Although little else is known of the amino acid sequences or molecular structure of the 190k protein, the probable similarity of its known region with regions found in ATP-dependent transport proteins suggests that it may also be a pump and therefore potentially able to be involved, either directly or indirectly, e.g. by establishment of pH or electrochemical gradients, with drug efflux. It has been shown that inhibitors of vacuolar H^+-ATPase can increase drug accumulation in MDR cells expressing either P-glycoprotein (HL60/Vinc) or a 190k protein (HL60/Adr) [22]. We have preliminary evidence that similar effects may be seen in L23/R cells (unpublished observations). Experiments are currently underway to determine if and in what way the vinca alkaloids may bind directly to the 190k protein and whether this binding is significant for drug efflux and cellular resistance.

Sensitisation to drugs and increases in drug accumulation in P-glycoprotein-containing MDR cells may be brought about by the resistance modifiers, cyclosporin A and verapamil. However in our non-P-glycoprotein-containing L23/R cells, cyclosporin A even at 5 μg/ml produced only very modest degrees of sensitisation to vincristine and to daunorubicin. This concentration of cyclosporin A is close to the IC_{50} for sensitiser alone

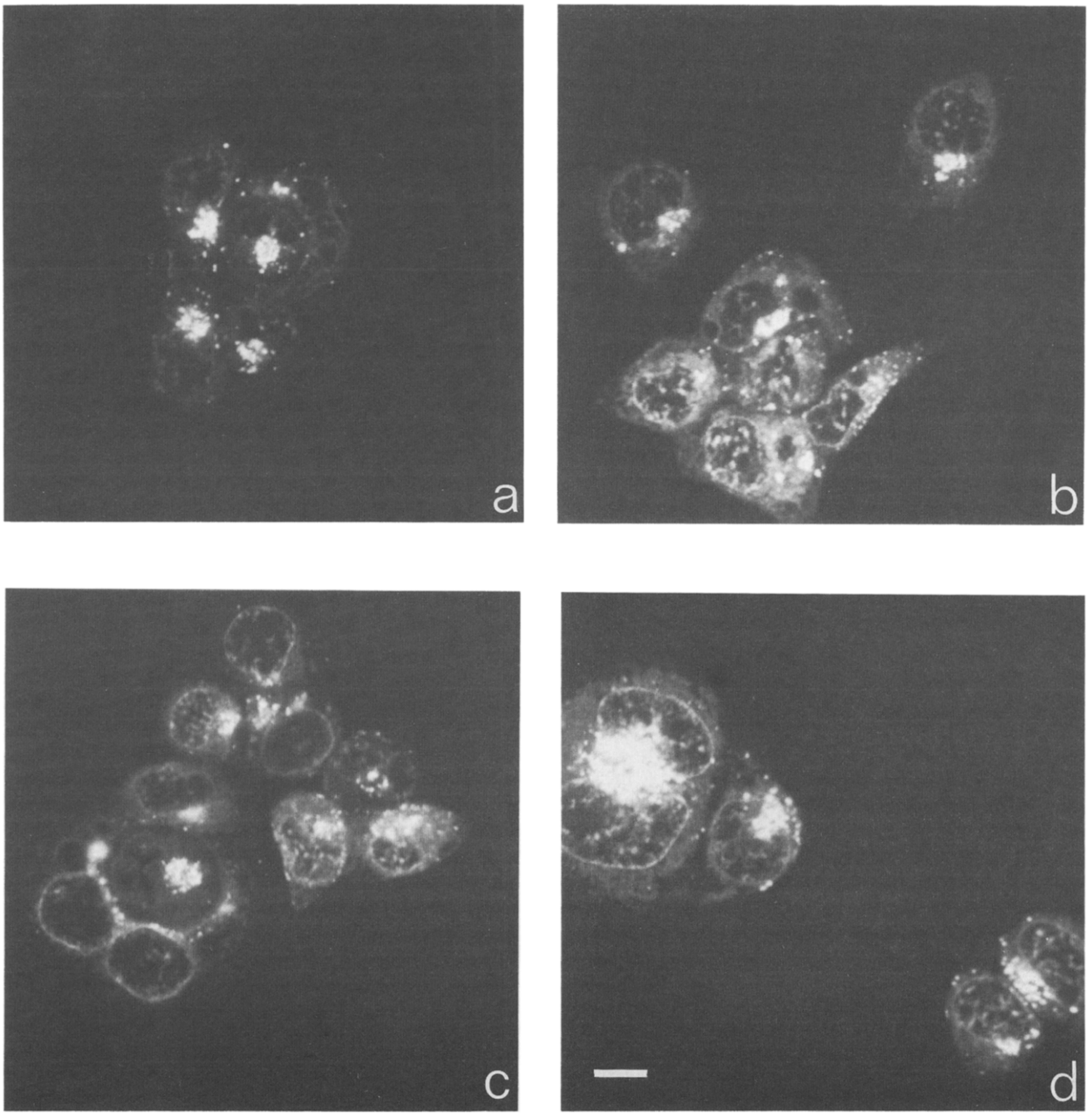

Fig. 5. Subcellular distribution of fluorescence in L23/R cells following exposure to daunorubicin at 1 μg/ml. Each photograph represents the average of images obtained from five scans. (a) With drug alone; (b) with drug plus cyclosporin A; (c) with drug plus PSC-833; (d) with drug plus verapamil. *Bar*, 10 μm.

estimated over a 6 day period [18] and hence is the highest possible concentration used in these studies. The sensitisation effects produced were not restricted to the resistant line alone so that the resistance factors were therefore only slightly reduced. This is in marked contrast to the sensitisation observed with the same doses of cyclosporin A in MDR cell lines which express P-glycoprotein [23], in several cases resistance being totally reversed. Particularly striking was the poor sensitisation response in our L23/R cells to the non-immunosuppressive cyclosporin analogue, PSC-833. It has previously been demonstrated in a variety of different MDR cell lines expressing P-glycoprotein that this analogue is 7–20 fold more potent than cyclosporin A at reversing resistance [15, 24]. In agreement with results of previous studies [7], verapamil at 3.3 μg/ml produced greater sensitisation effects in L23/R cells than in the sensitive parent L23/P cells thereby reducing the resistance factor. With each of the three modifiers examined, sensitisation to vincristine was greater than that to daunorubicin.

The resistance reversal effects of both cyclosporins and verapamil may result at least partly from their action on drug accumulation in P-glycoprotein-containing MDR cells in culture though alterations in drug sensitivity without concomitant changes in drug accumulation have been observed [10]. It has been suggested from photoaffinity labelling studies with analogues of cyclosporin and verapamil that these modifiers may act by binding to the drug efflux pump, P-glycoprotein in such a way

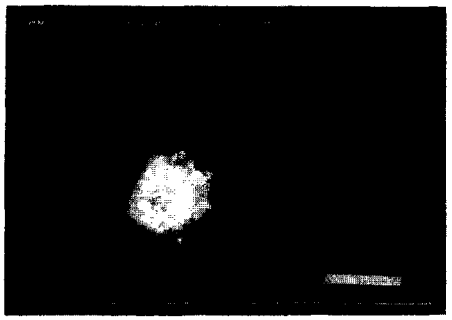

Fig. 6. Intracellular distribution of fluorescence in the Golgi apparatus of L23/R cells following exposure to fluorescent-labelled wheat germ agglutinin. *Bar*, 10 μm.

as to reduce its ability to expel cytotoxic drug from the resistant cells [10]. Despite the lack of P-glycoprotein in our L23/R cells, there is evidence of a drug accumulation deficit, a phenomenon thought to be associated with reduced drug efflux [7]. In these cells, cyclosporin A and PSC-833 were able to increase drug accumulation, yet despite this, these modifiers produced only a very modest degree of sensitisation to vincristine and daunorubicin. Verapamil at 3.3 μg/ml in fact increased the accumulation of vincristine in resistant cells to a level higher than that seen in the sensitive parent cells without modifier. Yet the same dose of verapamil could not restore sensitivity of the resistant cells to that of the parent cell line. Discrepancies between the chemosensitising effects of verapamil and its modulatory influence on drug accumulation have also been noted in P-glycoprotein-containing cells [25]. These discrepancies may serve to indicate that accumulation of drug observed over the course of hours may not be a good guide to the effects of the same drug on cell survival assayed over 6 days.

The relationship between resistance and drug accumulation is however complex and discrepancies between the level of drug accumulation and cellular sensitivity have repeatedly been demonstrated [26]. For example, similar levels of drug uptake have been observed in variants of a human squamous lung cancer cell line despite 4-fold differences in their sensitivity to doxorubicin [25]. Redistribution of drug away from sensitive target sites as opposed to overall decreased total cellular accumulation of drug may be the significant factor in determining resistance. In the present study, the distribution of daunorubicin fluorescence was markedly different in the resistant L23/R cells and in the sensitive parents L23/P cells with clear nuclear fluorescence in the sensitive cells only. In the resistant cells the fluorescent drug appeared to be excluded from the nucleus and was concentrated instead in perinuclear clusters of vesicles in the cytoplasm. Differences between sensitive and resistant cells in intracellular drug distribution have been reported previously, both in P-glycoprotein containing MDR cell lines and in those MDR cell lines lacking P-glycoprotein [5] with a gradual shift from a 'mainly nuclear' to a 'mainly cytoplasmic' distribution in MDR cells of gradually increasing resistance [25].

Resistance modifiers such as verapamil that reverse resistance in P-glycoprotein-containing MDR cells also induce redistribution of drug from cytoplasm to nucleus and presumably thereby increase the effectiveness of drug present within the cells [26, 27]. A small alteration in drug distribution was seen in our non-P-glycoprotein-containing MDR cells following treatment with cyclosporin A and PSC-833 with increases in both nuclear and cytoplasmic fluorescence sufficient to account for the changes seen in whole cell drug accumulation. Verapamil caused an increase particularly in nuclear staining but without modifying the intensely fluorescent vesicular component. Qualitative differences in the action of cyclosporin A and of verapamil have been observed also in some P-glycoprotein containing cells [28] with increases in sensitivity with verapamil reflected in a shift of fluorescence from the cytoplasm to the nucleus resulting in increases in DNA strand breaks but with cyclosporin A able to reverse resistance without bringing about very marked changes in drug distribution. It was suggested that cyclosporins may enhance drug toxicity partially by affecting mechanisms independent of the P-glycoprotein driven drug efflux. The interactions of cyclosporin A and verapamil with P-glycoprotein may be based on different mechanisms. Furthermore, other mechanisms not associated with cellular pharmacokinetics may also be involved in the sensitisation actions of cyclosporin A [11]. It has been observed that, despite interaction with P-glycoprotein, cyclosporin A though an effective inhibitor of daunorubicin transport does so without modifying cellular energy demands in the way seen with verapamil [29].

Compartmentalisation of drug away from its subcellular targets may be an important way in which resistance is achieved in some non-P-glycoprotein-containing cells. In our L23/R cells, the vesicular organelles which appear to concentrate the fluorescence drug show a distribution similar to that of the Golgi apparatus, as revealed by fluorescent labelled wheat germ agglutinin, a lectin which binds selectively to glycosylation sites on Golgi membranes [30]. We have not yet identified the subcellular location of the 190k protein in L23/R cells but it is interesting to note that the 190k protein hyperexpressed in the non-P-glycoprotein-containing MDR HL60/Adr cells appears primarily associated with the endoplasmic reticulum, only small amounts being detectable in the plasma membranes [17]. This could indicate a role for the protein in concentrating drug into vesicles. In another non-P-glycoprotein-containing MDR line (HL60/AR) derived from HL60 cells [5] but distinct from HL60/Adr, two phases of daunorubicin distribution have been distinguished, an initial accumulation in Golgi-like vesicles followed by an energy and temperature dependent shift to lysosomal elements [31]. It was suggested that exocytosis might be involved in the drug efflux mechanisms in this type of MDR cell.

Whatever mechanisms are responsible for resistance in our non-P-glycoprotein MDR cell line, COR-L23/R, it seems that these mechanisms may be relatively insensitive to the effects of some of those resistance modifiers that alter drug accumulation in P-glycoprotein-containing MDR cells and that are currently undergoing clinical trials as potential resistance reversal agents *in vivo* [11]. The exact role of the 190k protein in resistance in these cells is not yet established. The protein appears to be expressed also in MDR variants of several lung cell lines derived from tumours of different histological type [20] but it has yet to be determined how widely distributed this protein may be *in vivo*. However considering the insensitivity of MDR cells containing this protein to the sensitisation effects of some resistance modifiers, it seems important that the clinical relevance of this protein should be investigated. Should it prove to be an important component of clinical resistance, alternative 'resistance modification' strategies will need to be developed.

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